

The Regulation of Germ Cell
Proliferation in the Prepubertal
Marmoset Testis: Towards a Strategy
for Fertility Preservation in Boys
Treated for Cancer

A thesis submitted to the University of Edinburgh for the degree of
Doctor of Medicine.

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October 2006



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Declaration

I declare that this thesis has been composed by myself.

The material contained within this thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or qualification.

The work, of which this thesis is a record, has been undertaken by me unless otherwise specified within the text.

All sources of information have been acknowledged.

Thesis

Abstract

Background

Prepubertal boys treated for cancer may exhibit impaired fertility in later life. A number of chemotherapeutic agents have been identified as being gonadotoxic, and certain treatment regimens, such as that used for Hodgkin's disease, are particularly associated with subsequent infertility. Radiotherapy may also cause gonadal damage, most notably following direct testicular irradiation or total body irradiation.

Semen cryopreservation is currently the only method of preserving fertility in patients receiving gonadotoxic therapy. This is only applicable to postpubertal patients and can be problematic in the adolescent age group. At present there is no provision for the prepubertal boy, although there are a number of experimental methods currently being investigated. By harvesting testicular tissue prior to gonadotoxic therapy, restoration of fertility could be achieved following treatment, either by germ cell transplantation or by *in vitro* maturation of the germ cells harvested. An alternative, and potentially more acceptable method of fertility protection is to render the testes quiescent during cytotoxic treatment, thereby reducing the susceptibility of germ cells to subsequent damage.

However, attempts to protect gonadal function by suppression of the reproductive axis in primates have been unsuccessful, possibly because spermatogonial proliferation may be gonadotrophin independent. The primary aim of this project, using the marmoset monkey as an animal model, was to investigate whether spermatogonial

proliferation in this species is independent of gonadotrophins. Secondly, if this were to be confirmed, the aim was to identify factors that do regulate their proliferation, as a step towards devising strategies for fertility preservation in boys treated for cancer.

Methodology and Results

Immunohistochemistry was used on Bouins-fixed marmoset testes from birth to adulthood, including tissue from males treated prepubertally for ten weeks with a GnRH antagonist, to elucidate the role of gonadotrophins. No difference was found between germ cells from control and GNRH antagonist-treated animals in the immunoexpression of MAGE, a germ cell-specific marker, Ki67, a marker of cell proliferation, and Histone H3, a marker of cell mitosis, confirming that spermatogonial proliferation is gonadotrophin-independent.

To determine potential regulators, spermatogonial immunoexpression of growth factor receptors was investigated. Receptors for several growth factors were identified in spermatogonia. The expression of receptors for both Epidermal Growth Factor and Neurturin was pronounced in a proportion of germ cells in late infancy, thus implicating a role for these factors in spermatogonial development. Prepubertal expression of these receptors was unaffected by prior treatment with a GnRH antagonist, demonstrating their gonadotrophin independence. Studies using confocal microscopy demonstrated that the spermatogonial population expressing these receptors were not actively proliferating, raising the possibility that these factors act to inhibit proliferation. Manipulation of these factors may protect fertility in boys treated for cancer.

As survival of stem spermatogonia is essential for future fertility, this study also attempted to identify a marker of stem cells in the primate, in order to investigate their activity prepubertally. GFR- α 1, the receptor for Glial Cell Line-Derived Neurotrophic Factor, is expressed by A-spermatogonia in the mouse. In marmoset testis, expression of this receptor was shown in occasional germ cells, in a pattern consistent with stem cells, at all ages studied. Attempts to co-localise GFR- α 1 with cell cycle markers suggest these cells are not actively proliferating, but further studies are underway to fully investigate these cells, and their relationship with the growth factors already identified in this primate model.

Conclusions

These results confirm that spermatogonial proliferation in the prepubertal primate testis is gonadotrophin independent. These studies have also identified growth factor receptors expressed by spermatogonia during infancy, and have demonstrated that these are not regulated by gonadotrophins. In addition, a potential stem cell marker has been identified in the primate testis. Further investigation of these growth factors, and their relationship with stem cells within the testis, will contribute to the development of strategies to protect fertility in prepubertal boys undergoing cancer treatment.

Dedication

I dedicate this thesis to my father, Michael Brougham OBE.

Acknowledgements

I would like to express my huge thanks to all the people who have helped and supported me throughout this project. I am particularly indebted to my supervisors, Professor Richard Sharpe, Dr Hamish Wallace and Professor Chris Kelnar. Despite their extremely busy schedules they all provided me with tremendous support, encouragement and enthusiasm throughout. Whilst guiding me in the right direction, I was always given the freedom to explore and pursue my own ideas regarding the project. As such I feel very privileged that they have been my supervisors, and look forward to continuing work with them in the future.

I would also like to thank all those whom I worked with in the Reproductive Sciences Laboratory. I consider myself extremely fortunate to have worked in a laboratory that is so friendly and supportive. In particular I would like to mention Chris McKinnell and Nancy Evans, who both spent considerable time teaching me a variety of laboratory techniques. In addition I would like to thank Mike Millar and Sheila MacPherson for their never-ending ideas when experiments didn't quite go to plan!

I would also like to express my gratitude to both the Child Growth Foundation and Pfizer Pharmaceuticals, for their financial assistance.

Finally I would like to thank my wife, Karen, for her support, and my children, Rosie, Annabel and Millie.

Abbreviations

- **ALL** Acute Lymphoblastic Leukaemia
- **BMT** Bone Marrow Transplantation
- **BSA** Bovine Serum Albumin
- **DAB** 3,3-diaminobenzidine tetrahydrochloride
- **DMSO** Dimethylsulphoxide
- **DNA** Deoxyribonucleic acid
- **EGF** Epidermal Growth Factor
- **FGF** Fibroblast Growth Factor
- **FSH** Follicle Stimulating Hormone
- **GDNF** Glial Cell-line Derived Neurotrophic Factor
- **GFR** GDNF Family of Receptors
- **GM-CSF** Granulocyte Macrophage-Colony Stimulating Factor
- **GnRH** Gonadotrophin releasing Hormone
- **GnRHa** Gonadotrophin releasing Hormone antagonist
- **HRP** Horseradish Peroxidase
- **ICSI** Intracytoplasmic Sperm Injection
- **IGF** Insulin-like Growth Factor
- **IVF** In Vitro Fertilization
- **LH** Luteinizing Hormone
- **LIF** Leukaemia Inhibitory Factor
- **mRNA** Messenger Ribonucleic Acid
- **NBT** 4-nitro blue tetrazolium chloride

- **N-CAM** N-cell Adhesion Molecule
- **NRS** Normal rabbit serum
- **PCNA** Proliferating Cell Nuclear Antigen
- **RNAi** Ribonucleic Acid Interference
- **SCF** Stem Cell Factor
- **SIGN** Scottish Intercollegiate Guidelines Network
- **TBI** Total Body Irradiation
- **TBS** Tris-buffered saline
- **TGF** Transforming Growth Factor

Chapter 1

Introduction- Male Fertility following Childhood Cancer

1.1 Introduction

Survival from childhood cancer has markedly improved over the past few decades following major advances in available treatments and supportive care, such that now around 75-80% of children with cancer will be alive five years from diagnosis (Mertens *et al*, 2001). The number of long term survivors is therefore increasing, and it has been estimated that by the year 2010, about one in 715 of the adult population will have been treated for cancer in childhood (SIGN Guideline 76, 2004).

Because of this, the emphasis in the management of childhood cancer has changed from “cure at any cost” to one in which quality of life both during and after treatment has become increasingly important. Thus whilst continuing to strive for improved survival, attention must also be directed towards minimising the late effects of treatment.

Adverse late effects of childhood cancer treatment are diverse and include disorders of the endocrine system, cardiac and pulmonary dysfunction, renal and hepatic impairment, secondary malignancies and psychosocial difficulties.

Although problems with fertility do not become apparent until after puberty, it is clear that many treatments for childhood cancer can lead to infertility and sub-fertility in later life (Waring and Wallace, 2000). This can have a particularly devastating impact as the

patient enters adulthood. Having survived cancer as a child it can be very difficult for many such patients to accept that they cannot produce their own children because of the treatment they received earlier in their life. It is therefore imperative to consider, at an early stage, strategies that may protect or restore fertility in later life.

This chapter will discuss the aetiology of reduced fertility following treatment for childhood cancer in the male, and the ability to predict future fertility potential after particular treatment regimens. At present the options available to protect or restore fertility in this patient group are limited. However, there are a number of novel experimental strategies, which are currently generating much interest within the scientific community. The potential benefits and disadvantages of these strategies are outlined, before a more detailed discussion of one option, 'hormonal manipulation', which forms the basis of the work described in this thesis.

1.2 Anatomy and Physiology of the Testis

In order to appreciate the basis of male subfertility, both secondary to cancer treatment and from other causes, it is essential to understand testicular function. The adult testis fulfils two essential roles, the production and maturation of male gametes (spermatogenesis) and the synthesis and secretion of testosterone (steroidogenesis). Although the prepubertal testis does not complete these processes it is vulnerable to cytotoxic damage, and therefore testicular development during this period must be critical for normal function following puberty.

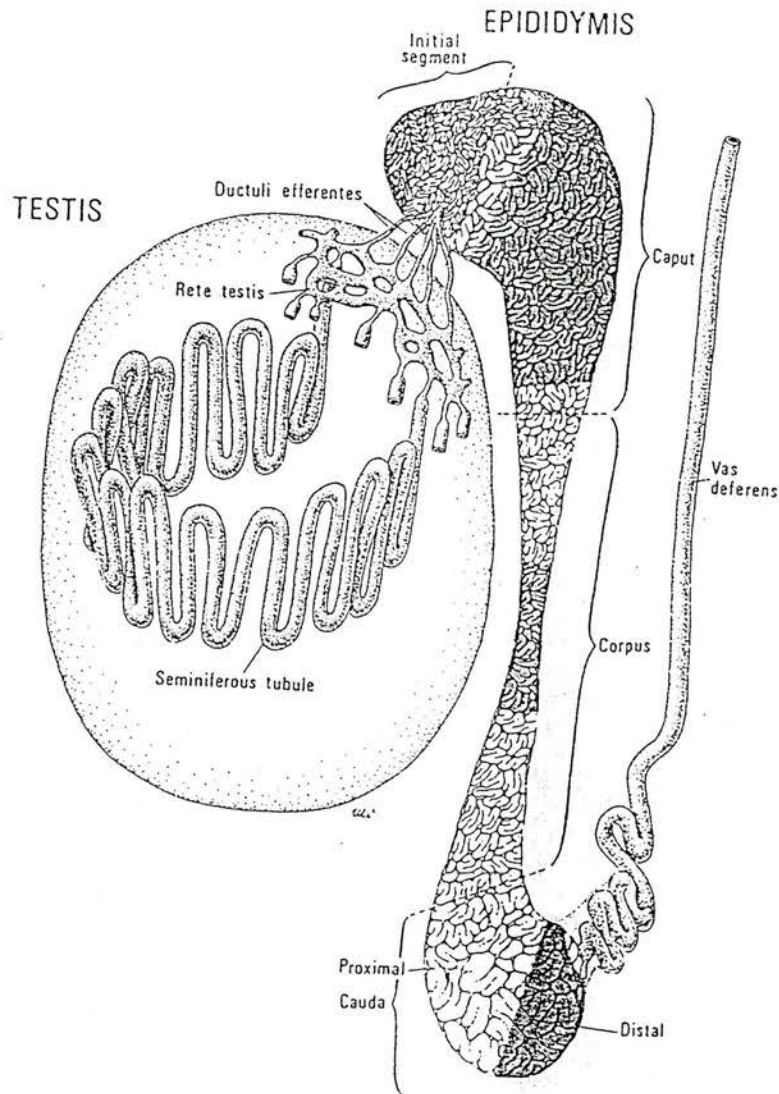
Spermatogenesis and steroidogenesis occur in two compartments of the testis which, although morphologically distinct, are functionally inter-related.

Testosterone is produced and secreted by the Leydig cells, located within the interstitial compartment. This testosterone both diffuses into the nearby seminiferous tubules, and enters the systemic circulation for distribution to its numerous target organs. In addition to Leydig cells, the interstitial area also contains macrophages, lymphocytes, blood and lymph vessels and connective tissue.

Spermatogenesis occurs within the seminiferous tubules. This compartment represents approximately 60-80% of the total adult testicular volume and, overall, the human testis contains about 600 seminiferous tubules. The wall of each tubule is composed of peritubular myoid cells that contract to cause peristalsis of the tubule (Maekawa et al, 1996), required for the transport of fully formed spermatozoa. Each end of the tubules is connected to the rete testis, which in turn connects to the epididymis via the efferent ductules (Fig 1.1). The seminiferous tubule contains two cell types, Sertoli cells and germ cells. The inter-relationship of these cell types within the epithelium of the tubules is essential for normal spermatogenesis, and will therefore be discussed in more detail.

Figure 1.1 Basic Anatomy of the Testis

Illustration of the anatomy of the testis, rete testis, efferent ducts, the regions of the epididymis and the vas deferens and their proximity to each other.



Adapted from Robaire and Hermo (1988)

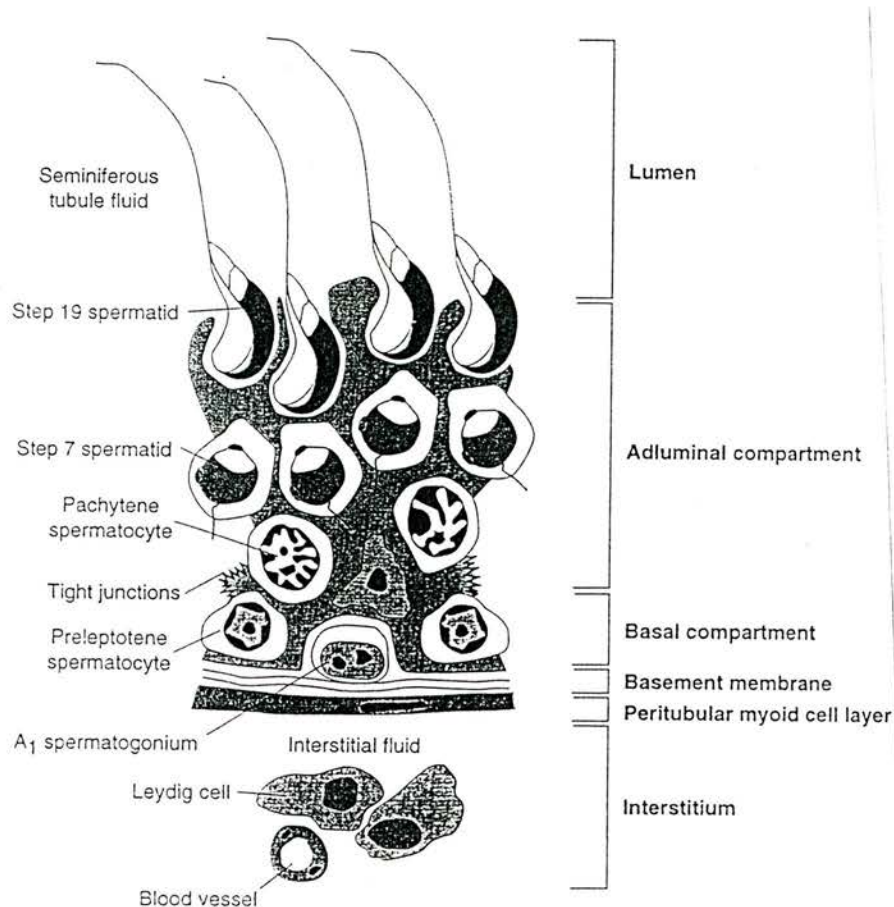
1.2.1 Sertoli Cells

Sertoli cells are somatic cells, and are responsible for nurturing the developing germ cells, from stem spermatogonia through to mature spermatozoa. They are located on the basement membrane, but their prominent cytoplasmic projections extend to the lumen of the tubule (Fig 1.2). This enables Sertoli cells to remain in both morphological and functional contact with the germ cells and, as demonstrated in Fig 1.2, these germ cells will be at different stages of development. Each Sertoli cell can only support a finite number of germ cells (Russell and Peterson, 1984), and therefore the number of Sertoli cells per testis will be an important factor in determining the number of sperm able to be produced.

Neighbouring Sertoli cells form inter-connecting tight junctions, which form the basis of the 'blood-testis barrier'. The diploid spermatogonia are located outside of this barrier in the 'basal compartment', whereas all meiotic and post-meiotic germ cells reside within the 'adluminal compartment', which is behind this barrier (Fig 1.2). It is thought that this barrier protects these haploid germ cells from immune recognition. However, because germ cells in this adluminal compartment are isolated, they do not have direct access to nutrients and hormones. They are therefore dependent on Sertoli cells for these requirements. In order to fulfil this role, Sertoli cells produce and secrete tubular fluid, which creates and maintains the patency of the tubular lumen (Griswold and Russell, 1999). This unique fluid immerses the germ cells, and presumably aids meiosis and sperm development. In addition to this fluid, Sertoli cells synthesise and secrete a large variety of factors including cytokines, growth factors and prostaglandins, which are likely

Figure 1.2 Illustration of a Sertoli cell with its associated Germ cells in the laboratory rat

(from Sharpe, 1994)



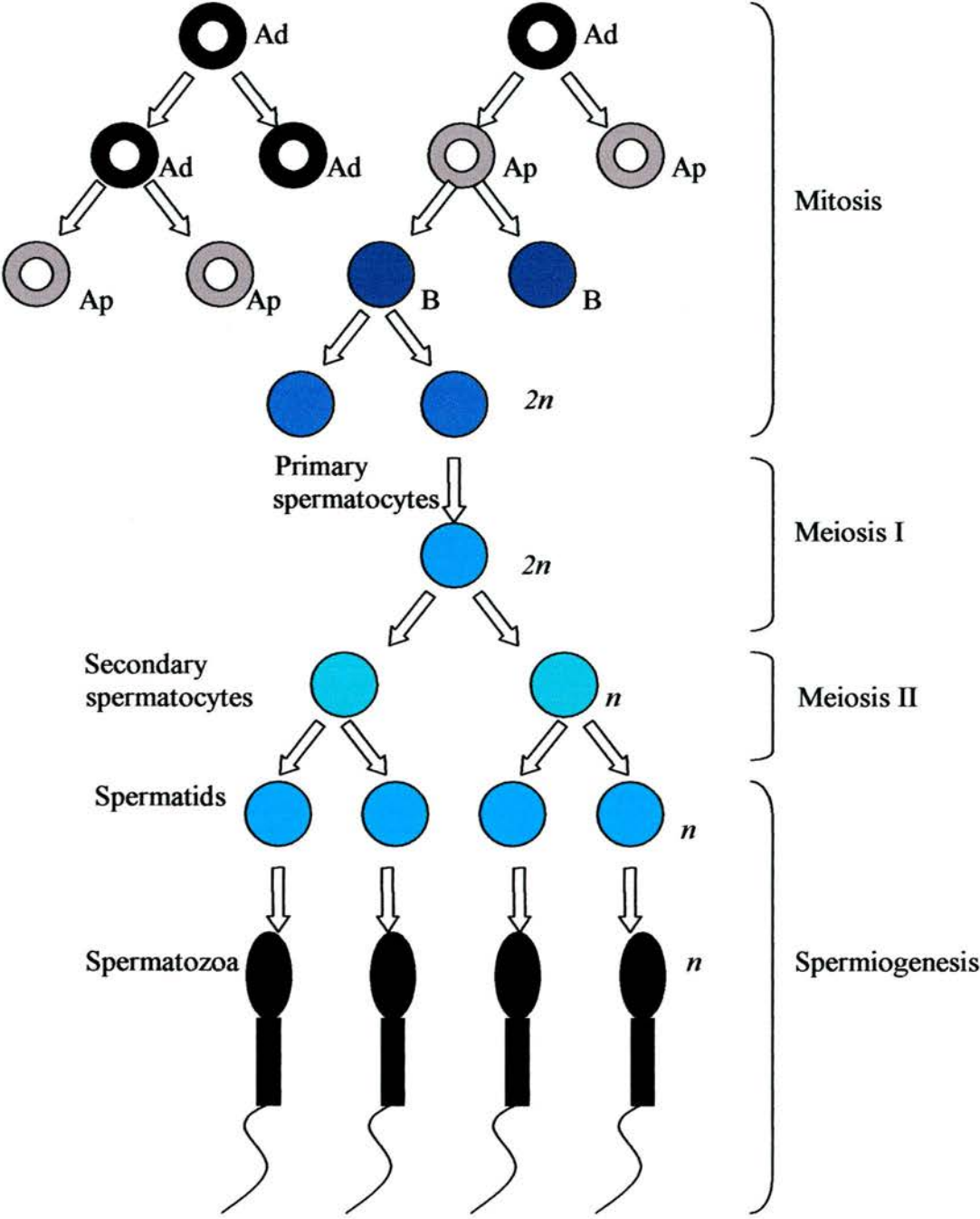
to act on the germ cells in a paracrine manner. Sertoli cells not only support developing germ cells, but are also responsible for phagocytosis of degenerating germ cells. In the human testis, as many as one third of germ cells degenerate by apoptosis (Johnson *et al*, 1984).

1.2.2 Germ Cells

Within the epithelium of the seminiferous tubule, germ cells mature from stem spermatogonia into spermatozoa capable of fertilisation. This maturational process, termed spermatogenesis, consists of three broad phases. Firstly, diploid spermatogonia undergo mitotic proliferation and differentiation to produce spermatocytes. Secondly, meiotic division of these spermatocytes results in the formation of haploid spermatids. Finally, these haploid germ cells transform into mature spermatozoa, predominantly via nuclear condensation with loss of cytoplasm and the formation of a flagellum. This final phase is termed spermiogenesis (Fig 1.3).

Spermatogonia are situated on the basement membrane of the seminiferous tubule, and are classified into two types, type A and type B. A-spermatogonia are further subdivided into Ad (dark) and Ap (pale), based on their cytological appearance following staining with haematoxylin (Clermont, 1966). Ad spermatogonia can be considered as 'reserve' stem cells. They are not proliferative under normal circumstances, but do become active when the number of Ap spermatogonia is reduced, for example after irradiation (van Alphen and de Rooij, 1986). In contrast, Ap spermatogonia divide once every epithelial cycle to produce B-spermatogonia (Sharpe, 1994). Mitotic division of B-spermatogonia

Figure 1.3 The Steps of Germ Cell Development in Human Spermatogenesis



gives rise to daughter cells that enter meiosis as preleptotene spermatocytes. These spermatocytes develop through leptotene, zygotene and pachytene phases to produce secondary spermatocytes. The final meiotic division of the latter cells subsequently produces four haploid round spermatids. These spermatids then undergo spermiogenesis to form mature sperm, which are released into the tubule lumen.

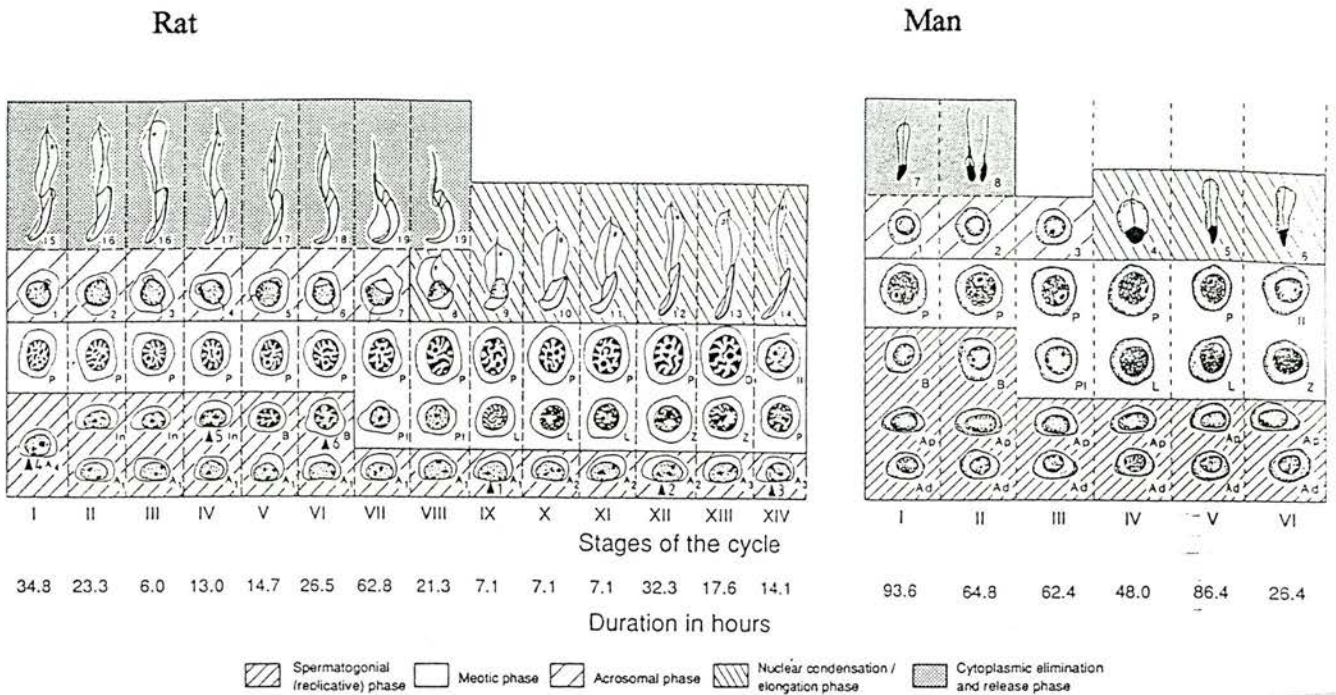
1.2.3 Organisation of Spermatogenesis

The progression of germ cells through this maturation process is ordered both in time and in space. As mentioned previously, Sertoli cells are in contact with a number of germ cells at different steps of this process. However, the different types of germ cell associated with one particular Sertoli cell is not random but is highly co-ordinated, in that cells at certain steps of maturation are only ever associated with cells at certain other steps. In this manner, spermatogenesis can be divided into certain stages, as demonstrated in Fig 1.4. Each stage represents the associations of germ cells at particular steps in the maturation process. When a germ cell completes the final stage at one level it subsequently progresses to stage I again, but at the next level, which becomes progressively more luminal. In this way each germ cell passes through the stages several times, before maturation into a spermatozoon.

Because Sertoli cells are in contact with germ cells at different steps of development, and those germ cells present are specific for particular stages of spermatogenesis, the function of Sertoli cells will change dependent on that particular stage. In other words, as germ cells progress through spermatogenesis, their requirements from the Sertoli cell will

Figure 1.4 Germ Cell Associations at the Different Stages of the Spermatogenic

Cycle in the rat (left) and in man (right) (from Sharpe, 1994)



Each vertical column or stage describes the fixed complement of germ cells associated with Sertoli cells at that particular stage, with the lumen at the top and the basement membrane to the bottom of the diagram. Each stage lasts a fixed period of time (indicated along the bottom), at the end of which each germ cell type within that stage will have developed into a germ cell characteristic of the following stage. The complete process of spermatogenesis can be divided into phases based on developmental events that occur. Thus there is an initial spermatogonial phase (A in rat, Ad and Ap in man) (arrowheads show when sequential mitoses occur), following which the B-spermatogonia (B) enter the meiotic phase as preleptotene spermatocytes (Pl) and subsequently develop through leptotene (L), zygotene (Z) and pachytene (P) primary spermatocyte stages before the final meiotic division (II, secondary spermatocyte), during which each spermatocyte gives rise to four haploid round spermatids. These then enter spermiogenesis, encompassing acrosome development, nuclear condensation, elongation and finally release of the spermatid, which on release is termed a spermatozoon.

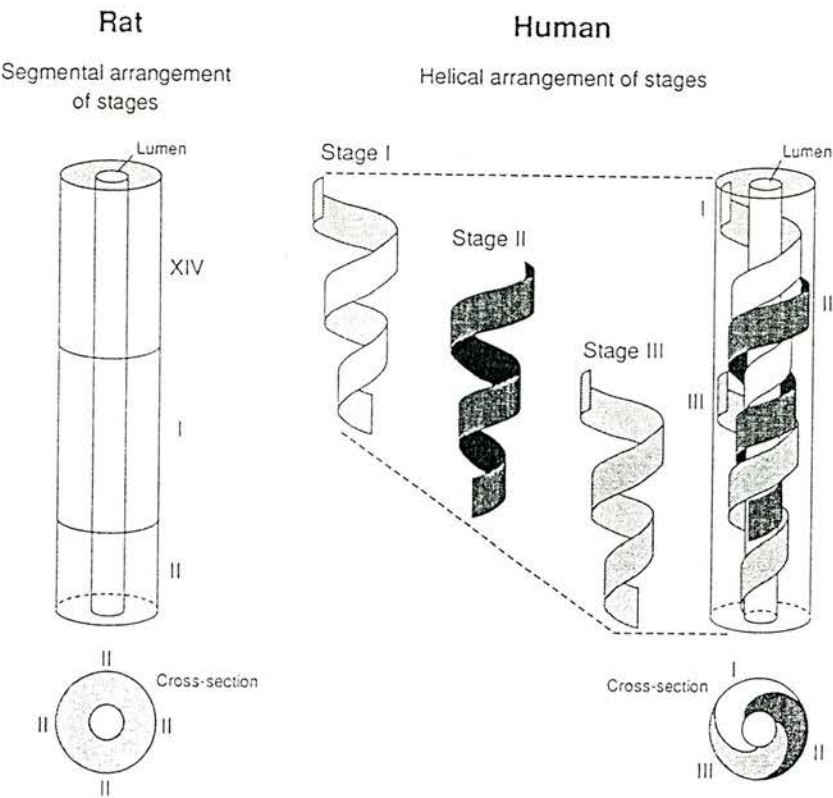
change accordingly. These cyclical changes in Sertoli cell function appear to be largely modulated by the germ cells themselves, with the particular complement of germ cells associated with the Sertoli cell determining its function (Parvinen, 1993).

The number of morphologically identifiable stages of the spermatogenic cycle differs depending on the species. The cycle in rats is classified into XIV stages, whereas in humans only VI can be distinguished. Each stage lasts a fixed period of time, and progression from stages I to VI lasts around $13\frac{1}{2}$ days in the human. Because each human germ cell passes through these stages $5\frac{1}{2}$ times, the overall duration of spermatogenesis lasts around 74-76 days (Sharpe, 1994).

The arrangement of these stages within the seminiferous tubule is also organised in a particular manner. In the rat, and indeed the majority of mammalian species, these stages have a 'segmental' arrangement along the tubule (Fig 1.5). Microscopic examination of a cross-section of such a tubule will demonstrate the presence of only one stage and, consequently, only the germ cell types present within that stage will be evident. In contrast, examination of the cross-section of a seminiferous tubule from the human demonstrates the presence of a number of stages within the same cross-section (Fig 1.5). Although this appearance initially suggests these stages are arranged randomly, more detailed examination suggests that there is a 'helical' arrangement of the different stages along the length of the tubule (Schulze *et al*, 1986). The great apes also exhibit a 'helical' arrangement of stages, but the majority of primates used widely for research, such as the rhesus monkey, exhibit a 'segmental' pattern (Sharpe, 1994). In contrast, the

Figure 1.5 The Arrangement of the Stages of Spermatogenesis along the Seminiferous Tubule in the Rat and in Man

(from Sharpe, 1994)



marmoset exhibits a pattern that is somewhat intermediate between ‘segmental’ and ‘helical’, though is generally quite similar to the human (Miller *et al*, 2000).

Because a number of different stages of spermatogenesis are present within a cross-section of a tubule from animals with a helical arrangement, interpretation of these tubules under a microscope can be more problematic than examination of rodent testes. More importantly, animals with a segmental arrangement are associated with highly efficient spermatogenesis. In contrast humans, and other animals known to exhibit a helical arrangement of spermatogenesis, are recognised as inefficient. It is uncertain whether this latter arrangement causes the inefficiency, or alternatively that inefficient spermatogenesis leads to the development of a helical pattern (Schulze and Salzbrunn, 1992). However, this highlights an important difference between spermatogenesis in rodents as compared to humans and certain other primates, and illustrates that there are limitations in extrapolating data regarding spermatogenesis between these species.

1.2.4 The Regulation of Spermatogenesis

The hypothalamic-pituitary-gonadal axis has an essential role in regulating spermatogenesis in the post-pubertal testis. However, given that post-meiotic germ cells are isolated from circulating hormones by virtue of the blood-testis barrier, the main aspects of this control are indirect, predominantly via Sertoli cells. Thus a combination of endocrine, paracrine and perhaps autocrine factors are likely to be involved.

1.2.4.1 Hormonal Control of Spermatogenesis

For normal spermatogenesis to occur, the testis requires stimulation by the pituitary gonadotrophins, the secretion of which is regulated by gonadotrophin-releasing hormone (GnRH) from the hypothalamus. Follicle Stimulating Hormone (FSH) acts directly on the seminiferous epithelium, whereas Luteinizing Hormone (LH) exerts its influence on spermatogenesis by stimulating testosterone secretion from the Leydig cells. Both gonadotrophins are under negative feedback control, with testosterone inhibiting their secretion. In addition inhibin B, produced by the Sertoli cell, is involved in the negative feedback control of FSH (Anderson and Sharpe, 2000).

Matsumoto and Bremner (1989) induced gonadotrophin deficiency in normal men by the administration of testosterone at high doses, resulting in oligospermia or azoospermia. Sperm counts increased on selective replacement of either LH activity or FSH activity, but remained below those within the control group. Thus normal levels of both FSH and LH are required to achieve quantitatively normal sperm production in man.

Although both FSH and testosterone appear to be essential for normal spermatogenesis, the precise role of both these hormones in the testis is uncertain. Germ cells do not possess receptors for either FSH or testosterone. However, Sertoli cells express receptors for both hormones, and androgen receptors are also present on peritubular cells (Bremner *et al*, 1994). These cells therefore act as intermediaries, with FSH and testosterone acting on germ cells in an indirect manner.

Testosterone is essential for the maintenance of normal spermatogenesis in the post-pubertal testis. This hormone acts in both an endocrine and paracrine manner, as not only circulating levels must be adequate, but also intratesticular transport of testosterone is essential for complete spermatogenesis in mice (Takaimiya *et al*, 1998). Indeed, intratesticular levels of testosterone must be sufficient for normal spermatogenesis to occur in both rodents and primates (Sharpe, 1994). In addition, testosterone also has a role in spermiogenesis and spermiation (McLachlan *et al*, 2002).

As explained previously, androgen receptors are not present on germ cells, and therefore testosterone presumably acts via the Sertoli cell, and perhaps the peritubular cell.

Withdrawal of testosterone in the rat results in germ cell degeneration, but only at certain stages of the spermatogenic cycle (Dym and Raj, 1977; Sharpe, 1994). Thus testosterone may act only upon certain stages of spermatogenesis, rather than having a more general effect (Sharpe, 1994). This stage-dependent regulation reflects the intimate coupling of Sertoli cell function and germ cell maturation.

The role of FSH in adult spermatogenesis is difficult to elucidate, in part because of apparent differences between rodent models and primates. Immunoneutralization of endogenous FSH has little or no impact on spermatogenesis in rats (Davies *et al*, 1979), whereas in non-human primates it can lead to a significant suppression of spermatogenesis and sperm output (Wickings and Nieschlag, 1980). Indeed, FSH has been shown to play a role in the progression of type A to type B spermatogonia in primates (McLachlan *et al*, 2002). In contrast, regulation of spermatogonial replication

and differentiation in rodents is not mediated primarily by FSH, but more by density-dependent regulatory factors (Sharpe, 1994). This relates to the higher number of spermatogonial cell divisions exhibited by the rodent, resulting in the number of B-spermatogonia entering meiosis being maintained at maximal levels. In primates the number of B-spermatogonia is well below these levels. This further highlights significant differences between spermatogenesis in rodents and in certain primates, particularly with regard to overall efficiency.

Although FSH on its own may not appear as significant as testosterone in the regulation of spermatogenesis, especially in rodents, it does exhibit synergistic action with testosterone, particularly with regard to the regulation of germ cell viability (McLachlan *et al*, 2002). FSH increases the availability of germ cells at certain steps of development for entry into the androgen dependent phases of the spermatogenic cycle (Sharpe, 1994).

As with testosterone, these FSH-mediated effects are likely to be via the Sertoli cell, as germ cells do not possess FSH receptors. Thus interaction between the Sertoli cell and germ cells is vital and, as mentioned previously, the changing germ cell complement from stage to stage determines the alterations in hormonal responsiveness of the Sertoli cell (Parvinen, 1993).

1.2.4.2 Local Control of Spermatogenesis

Whilst spermatogenesis is regulated in part by systemic factors, in particular FSH, it is clear from the above discussion that regulation also exists within the testis at a local level.

Factors produced locally are likely to be important in the modulation of hormonal activity, particularly at different stages of the spermatogenic cycle.

Firstly, testosterone, produced by the Leydig cell, has important actions locally in the seminiferous tubules. As discussed above, an adequate level of intratesticular testosterone is essential for the maintenance of normal spermatogenesis.

Secondly, as mentioned previously, each Sertoli cell can only support a fixed number of germ cells to maturity, and therefore the number of Sertoli cells present will determine the level of potential sperm output. In the rat, FSH is the most important regulator of Sertoli cell replication (van den Dungen *et al*, 1990), and this ceases during neonatal life with Sertoli cell maturation and the formation of tight junctions between adjacent Sertoli cells (Sharpe *et al*, 2003a). A similar situation exists in man, although a degree of Sertoli cell replication may continue throughout the prepubertal period (Cortes *et al*, 1987; Sharpe *et al*, 2003a).

It is clear that both FSH and testosterone have essential roles in spermatogenesis. However, an increasing number of growth factors and cytokines have been identified within the testis, and these are also likely to be important with regards to testicular function. The main emphasis of this project is the identification of these factors in the primate testis, and as such will be discussed in more detail later on.

1.2.5 Testicular Development

Whilst much of the preceding discussion relates to the adult testis, the patient group around which this project focuses is prepubertal. It is therefore appropriate to discuss development of the testis, and the functional changes that occur through foetal, neonatal and prepubertal phases of maturation.

1.2.5.1 Foetal and Neonatal Development

The testis exhibits a number of critical phases of development during foetal and neonatal life. As there is considerable overlap in the timing of these phases, both periods will be considered together.

The embryonic testes develop from the indifferent gonads, which are mesoderm-derived structures located either side of the dorsal aorta and neural tube. Differentiation into male gonads occurs at around 7 weeks of foetal life in the human, directed by the expression of the testis-determining SRY gene on the Y chromosome (Gubbay *et al*, 1990).

The indifferent gonad consists of four major cell types, reflecting those cells present in the mature testis. Firstly, the supporting cell lineage gives rise to Sertoli cells in males. As discussed previously, proliferation of these cells in both foetal and neonatal life is an important determinant of future sperm production. Although FSH is important in the postnatal regulation of Sertoli cell replication, it has been demonstrated using murine models that foetal development of Sertoli cells is independent of gonadotrophins (Baker

and O'Shaughnessy, 2001), and may instead be androgen-dependent (Johnston *et al*, 2004).

Secondly, cells from the steroidogenic lineage are the precursors of Leydig cells. During normal testicular development, two separate populations of Leydig cells arise in a sequential manner. The 'foetal population' is present soon after testicular differentiation, and these cells produce testosterone, required for masculinization of the male foetus. In humans, unlike rodents, this population exhibits a further wave of activity causing a testosterone peak in the neonatal period, the role of which remains uncertain (Mann and Fraser, 1996). The 'adult population' of Leydig cells develop later, and are responsible for masculinization at puberty, and its maintenance throughout adulthood (Saez, 1994).

Thirdly, the connective cell lineage gives rise to the peritubular myoid cells in the male. These cells migrate from the mesonephros into the developing gonad (Brennan and Capel, 2004).

The fourth important cell type present is the germ cells themselves, and these begin as Primordial Germ Cells, located in the endoderm of the yolk sac. These cells migrate into the gonad by the 6th week of foetal life and become surrounded by Sertoli cells (Brennan and Capel, 2004). Subsequently these germ cells, now known as gonocytes, migrate towards the basement membrane of the primitive seminiferous tubule. Pseudopod development, required for this migration, is dependent on the presence of c-kit, the receptor for Stem Cell Factor, which is expressed by gonocytes during the foetal and

neonatal period (Orth *et al*, 1997). Other factors are also likely to play a role in this migration, including cell adhesion molecules, such as N-CAM (Orth *et al*, 1995). It should be emphasised, however, that these findings are based solely on rodent models, rather than primate species. Gonocytes are able to reach the basement membrane because tight junctions between adjacent Sertoli cells are not fully formed at this stage. Having reached this site germ cells are known as spermatogonia, and will include a subset of cells that will provide the stem cells for future spermatogenesis.

1.2.5.2 The Prepubertal Phase of Testicular Development

It is apparent that the foetal and neonatal periods of testicular development are critical for normal function, and that the adult testis is a highly active organ responsible for spermatogenesis and testosterone production. In contrast, the prepubertal testis has traditionally been thought of as a quiescent organ, with little or no significant cellular activity.

Chemotherapy and radiotherapy target rapidly dividing cells and therefore, if the prepubertal testis is quiescent, this organ should not be susceptible to such cytotoxic damage. However, it is clear that cancer treatment given to prepubertal boys can impair future fertility (Whitehead *et al*, 1982; Relander *et al*, 2000), indicating that the testis is susceptible to such damage in this age group.

The majority of studies investigating testicular function are based on rodent models, and because the prepubertal phase of development is relatively short in these species,

knowledge regarding this period has been limited. However, with the use of primate models, there is increasing evidence that far from being a quiescent organ, the prepubertal testis demonstrates significant cellular activity. This activity includes a steady turnover of early germ cells, which undergo spontaneous degeneration before the haploid stage (Rey *et al*, 1993). In addition, as mentioned previously, Sertoli cells continue to proliferate during this phase of development. It is postulated that this activity is essential for normal adult function (Chemes, 2001), and therefore disruption of these processes may affect fertility as an adult.

Therefore, if this activity also occurs in the prepubertal human testis, cytotoxic treatment received during this time could affect gonadal function, and this might explain the effects on fertility observed in adulthood. This project focuses on the investigation of prepubertal testicular activity, and as such will be discussed throughout this work.

1.3 Gonadal Toxicity following cancer treatment

Gonadal damage in boys treated for cancer can result from either systemic chemotherapy or radiotherapy involving the spinal or pelvic area. This damage may involve both the somatic cells of the testis, the Sertoli and Leydig cells, as well as the germ cells.

Because cytotoxic treatment, by its nature, targets rapidly dividing cells it is not surprising that spermatogenesis can be impaired after treatment for cancer. The exact mechanism of this damage is uncertain but appears to involve depletion of the proliferating germ cell pool, by killing cells not only at the stage of differentiating

spermatogonia (Meistrich *et al*, 1982) but also stem cells themselves (Bucci and Meistrich, 1987). In addition, stem spermatogonia that do survive fail to differentiate further (Kangasniemi *et al*, 1996). Although the prepubertal testis does not complete spermatogenesis and produce mature spermatozoa, as discussed previously the testis does demonstrate significant cellular activity at this age. Thus the timing of the gonadotoxic insult in relation to puberty is of less importance than the nature of the insult itself.

1.3.1 The Effects of Chemotherapy

The nature and extent of damage to the testis from cytotoxic chemotherapy is dependent upon the agent administered, the dose received and the age of the patient (Watson *et al*, 1985; Wallace *et al*, 1989; Wallace *et al*, 1991; Mackie *et al*, 1996). Since nitrogen mustard was first linked to azoospermia by Spitz in 1948 (Spitz, 1948), a number of chemotherapeutic agents have been identified as causing long-lasting or permanent gonadotoxicity. These include the alkylating agents such as chlorambucil, cyclophosphamide and melphalan, anti-metabolites such as cytarabine, and others including procarbazine and cis-platin. Examples of gonadotoxic chemotherapy agents used in recent years are listed in table 1.1, although it is emphasised that this is not intended as a comprehensive list. However, it is important to note that not all chemotherapeutic agents are associated with subsequent gonadal dysfunction. In addition, most of these agents are given as part of multi-agent treatment regimens, and thus the relative contribution of each individual drug can be difficult to determine.

Table 1.1: Gonadotoxic Chemotherapy Agents

Alkylating Agents	Cyclophosphamide
	Ifosfamide
	Nitrosoureas e.g. carmustine, lomustine
	Chlorambucil
	Melphalan
	Busulphan
Vinca-alkaloids	Vinblastine
Anti-metabolites	Cytarabine
Others	Cisplatin
	Procarbazine

Within the testes, the seminiferous epithelium is the area most sensitive to the detrimental effects of chemotherapy. Therefore, after receiving gonadotoxic agents, patients may be rendered oligospermic or azospermic but testosterone production by the Leydig cell is usually unaffected, and thus secondary sexual characteristics develop normally (Kreuser *et al*, 1987; Thomson *et al*, 2002a). Following higher, cumulative doses of gonadotoxic chemotherapy, Leydig cell dysfunction may also become apparent (Gerl *et al*, 2001).

The impact of chemotherapy on testicular function has been extensively studied in patients treated for Hodgkin's disease. Treatment of this lymphoma has involved the use of procarbazine and alkylating agents such as chlorambucil, mustine and cyclophosphamide. Whilst this treatment leads to excellent survival rates, the majority of male patients have subsequently developed permanent azoospermia (Kreuser *et al*, 1987). Mackie *et al* (1996) studied children who had received treatment with 'ChlVPP', a chemotherapy regimen containing chlorambucil and procarbazine. The mean age at diagnosis of the male patients investigated was 12.2 years, and of these 89% subsequently had evidence of severe damage to the seminiferous epithelium up to ten years following therapy. This study demonstrates both the gonadotoxicity of these agents, and also the susceptibility of the prepubertal testis. Whitehead *et al* (1982) similarly followed up children treated with 'MOPP' chemotherapy, a regimen containing mustine and procarbazine, and also demonstrated subsequent long-term testicular damage in the majority of male patients. In view of these studies, treatment for Hodgkin's disease has been modified in an attempt to reduce the gonadotoxicity, whilst maintaining long-term survival (Thomson and Wallace, 2002).

One such modification is a reduction in the dose of procarbazine and alkylating agents, and the addition of anthracycline agents such as adriamycin which, although potentially cardiotoxic, do not permanently affect spermatogenesis. This hybrid regimen, known as ChlVPP/EVA and consisting of seven different chemotherapeutic agents, has been compared to a standard regimen similar to 'MOPP' (Clark *et al*, 1995). However, this hybrid regimen was found to be equally gonadotoxic, with azoospermia resulting in 95% of men following treatment.

Gonadal damage may be lessened by removing alkylating agents and procarbazine altogether, such as with the 'ABVD' regimen (adriamycin, bleomycin, vinblastine and dacarbazine). This protocol is significantly less gonadotoxic than 'MOPP' chemotherapy, as demonstrated by Viviani *et al* (1985). In this study cohort, treatment with 'ABVD' resulted in temporary azoospermia in 33% of patients and oligospermia in 21%, with recovery of spermatogenesis observed in all patients after 18 months. In contrast, 97% of patients were azoospermic following 'MOPP' chemotherapy, and in the majority this damage was permanent.

In the United Kingdom at present, treatment of Hodgkin's disease involves combination chemotherapy, with courses of chemotherapy that alternate between those containing alkylating agents with those containing anthracyclines. Currently this involves 'OEPA' (vincristine, etoposide, prednisolone and adriamycin) alternated with the potentially gonadotoxic 'COPP' (cyclophosphamide, vincristine, procarbazine and prednisolone). Although this exposes the child to a wide variety of drugs it is hoped that excellent

survival rates will be obtained with minimal long-term adverse effects. Indeed, this type of regimen for Hodgkin's disease results in significantly less gonadotoxicity than does therapy based on alkylating agents and procarbazine alone (Anselmo *et al*, 1990; Longo *et al*, 1997), with approximately half of all male patients preserving their fertility. However, treatment will continue to be modified in order to optimise the long-term future for children with Hodgkin's disease.

1.3.2 The Effects of Radiotherapy

The gonads are sensitive to radiotherapy, and the subsequent damage depends on the field of treatment, total dose and fractionation schedule (Speiser *et al*, 1973; Rowley *et al*, 1974; Clifton and Bremner, 1983; Centola *et al*, 1994). Fractionation usually improves the therapeutic margin, but there is evidence to suggest that the gonads are an exception (Ash, 1980), and that fractionation may actually be more harmful to testicular function.

It has been demonstrated that doses as low as 0.1-1.2Gy can have detectable effects on spermatogenesis in adult men (Clifton and Bremner, 1983; Centola *et al*, 1994), with doses over 4Gy causing a more permanent detrimental effect (Speiser *et al*, 1973; Centola *et al*, 1994). As with chemotherapy, somatic cells are more resistant to radiation induced damage than are germ cells. Indeed, Leydig cell dysfunction is not observed until doses of around 20Gy are administered to the prepubertal boy, and up to 30Gy in sexually mature males (Shalet *et al*, 1989; Castillo *et al*, 1990). Testosterone production is therefore relatively preserved below these doses, and thus many patients will develop normal secondary sexual characteristics, despite severe impairment of spermatogenesis.

Within paediatric oncology, radiation induced gonadal damage is most often encountered following direct testicular irradiation, as used for management of testicular relapse of leukaemia, or following total body irradiation (TBI) given prior to bone marrow transplantation (BMT).

Radiation doses of 24Gy are usually used to treat testicular involvement in leukaemia, and this results in permanent azoospermia (Castillo *et al*, 1990). The effects of TBI on gonadal function can be difficult to elucidate as this is usually given with other treatment modalities, including high dose chemotherapy. However, doses of 9-10Gy have been associated with subsequent gonadal failure (Leiper *et al*, 1987), and gonadal damage is certainly more likely than in patients treated with chemotherapy alone (Liesner *et al*, 1994).

Tumours of the central nervous system are the commonest solid malignancy seen in the paediatric population. Cranial irradiation is frequently used as a therapeutic modality in these children. Whilst not harming the gonads directly, fertility can be affected by disruption to the hypothalamic-pituitary-gonadal axis. Indeed, patients receiving radiation doses of 35-45Gy have demonstrated subsequent deficiencies in FSH and LH secretion (Littley *et al*, 1989). The clinical sequelae of gonadotrophin deficiency exhibit a broad spectrum of severity, from subclinical abnormalities detectable only by GnRH testing, to a significant reduction in circulating sex hormone levels and delayed puberty.

It has been demonstrated, at least in female patients, that the aetiology of hypogonadism following cranial irradiation is hypothalamic GnRH deficiency (Hall *et al*, 1994). Thus exogenous GnRH can be used as replacement therapy in order to restore gonadal function and fertility.

1.3.3 The Effects of Disease

Although many aspects of cancer treatment may affect fertility, it is important to note that the disease itself may contribute to male gonadal dysfunction. Indeed, it has been demonstrated that up to 70% of patients with Hodgkin's disease assessed prior to commencing treatment have impaired semen quality (Rueffer *et al*, 2001). This has also been shown with other malignancies (Hallak *et al*, 2000), although perhaps not to the same extent (Botchan *et al*, 1997). In addition to the disease itself, other non-specific conditions commonly observed at presentation, such as fever, anorexia and pain, can impair semen quality (Agarwal *et al*, 1996). These findings on semen quality prior to treatment have only been investigated in adult patients, as similar studies on pre-pubertal boys cannot be performed. However, the results may have implications when options for preserving fertility in this patient group become technically and clinically feasible.

1.4 The Potential for Fertility following Cancer Treatment

Because of the varied nature of the gonadal insult following chemotherapy or radiotherapy, it can often be difficult to predict whether a child undergoing cancer treatment will subsequently have impaired fertility as an adult. The risk of subfertility can be categorised according to the type of malignancy and associated treatment (Table

Table 1.2: Best assessment of risk of subfertility following current treatment for childhood cancer by disease

Risk of subfertility	Disease/Treatment
Low	Acute lymphoblastic leukaemia Wilms' tumour Soft tissue sarcoma: stage 1 Germ cell tumours (with gonadal preservation and no radiotherapy) Retinoblastoma Brain tumour: Surgery only Cranial irradiation<24Gy
Medium	Acute myeloblastic leukaemia Hepatoblastoma Osteosarcoma Ewing's sarcoma Soft tissue sarcoma Neuroblastoma Non-Hodgkin's lymphoma Hodgkin's disease: 'alternating therapy' Brain tumour: Craniospinal radiotherapy Cranial irradiation>24Gy
High	Total body irradiation Localised radiotherapy: pelvic/testicular Chemotherapy conditioning for bone marrow transplant Hodgkin's disease: alkylating agent based therapy Soft tissue sarcoma: metastatic

Low risk is assessed at <20%, high risk as >80%. Medium risk is difficult to quantify. Males are more susceptible to subfertility following chemotherapy than females, although females may be at risk of premature menopause. It is emphasized that this table represents a guide only, and is based on gonadotoxicity of agents involved along with long term follow up of patients. In addition, the list of diagnoses is not fully comprehensive, due to the variability of disease sub-types and stage at diagnosis.

1.2). As can be seen, treatment for Hodgkin's disease with alkylating agent-based therapy is profoundly gonadotoxic, as discussed above. Conditioning prior to bone marrow transplantation, with high dose chemotherapy and total body irradiation, also carries a substantial risk of gonadotoxicity, as do treatment of metastatic sarcoma and testicular irradiation. In contrast, current treatment for Acute Lymphoblastic Leukaemia (ALL), the commonest malignancy in childhood and adolescence, represents a relatively low risk of gonadotoxicity.

It is important to note that treatment protocols for malignant disease are continually evolving, in order to improve survival and reduce adverse effects. Whereas treatments for ALL and neuroblastoma have intensified over the past decade, the management of Non-Hodgkin's Lymphoma and hepatoblastoma, for example, has become less intensive. In addition, as more is known about the biology of malignant disease, there is an increasing trend to stratify treatments according to risk of relapse. Therefore, this assessment of risk represents a guide, which needs to be continually reviewed in the light of ongoing research.

To complicate matters further, there are reports of patients having received sterilizing treatment who have subsequently demonstrated recovery of spermatogenesis (Marmor and Duyck, 1995). This not only has implications for counselling with regards to infertility, but also demonstrates the importance of discussing contraception with adult patients whose fertility status is uncertain.

In view of these uncertainties, informing children and their families about future fertility prospects can be very difficult. Long term follow-up of these patients must include appropriate discussion and assessment of fertility.

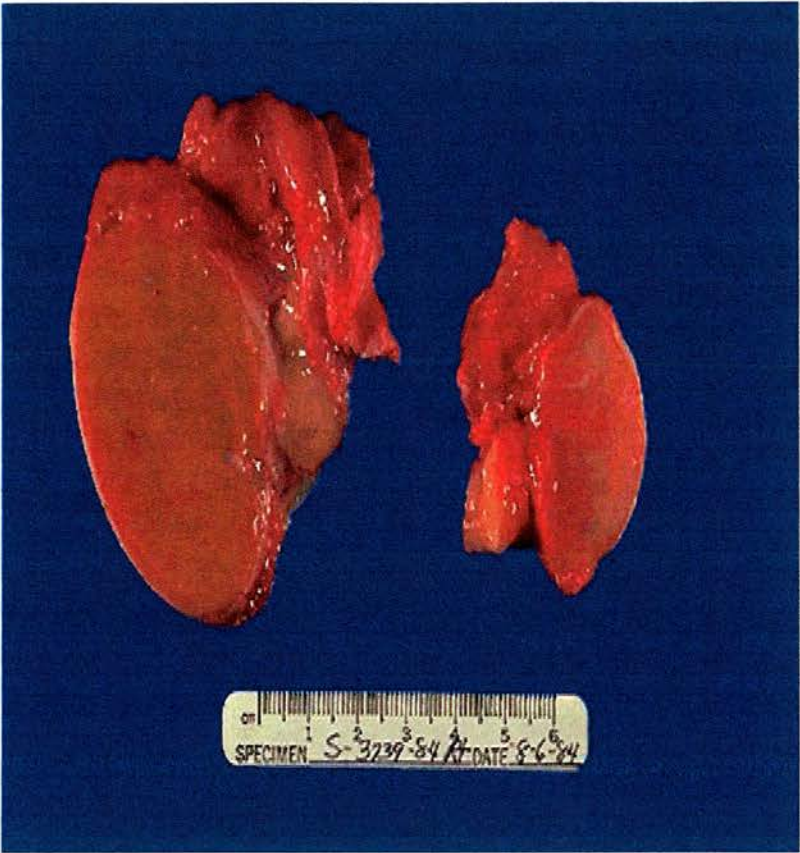
Determining the impact of cancer treatment on gonadal function currently involves regular clinical assessment of pubertal status, biochemical assessment of plasma gonadotrophins and testosterone, and analysis of semen specimens.

Testicular enlargement is the initial sign of puberty in boys, followed by penis enlargement and the development of pubic hair (Tanner, 1962). As discussed earlier, many male patients following gonadotoxic therapy will have preserved Leydig cell function and therefore develop normal secondary sexual characteristics. Their testes, however, will be small and atrophied, with a loss of tubular volume (Fig 1.6), suggestive of reduced sperm production (Siimes and Rautonen, 1990). On clinical examination, using the Prader orchidometer, a testicular volume of 12ml or less in a postpubertal male is likely to be associated with azoospermia.

However, in prepubertal children, clinical assessment such as this is non-contributory and biochemical assessment is unreliable because the hypothalamic-pituitary-gonadal axis is relatively quiescent in this age group. Thus it is currently not possible to detect gonadal damage early, due to the lack of a sensitive marker of gonadal function in prepubertal children.

Figure 1.6 Small, Atrophied Testis following Gonadotoxic Therapy

Normal testis on left, with atrophied testis following gonadotoxic therapy on the right



There is currently much interest in plasma inhibin B as a potential marker of gonadotoxicity in this age group. Inhibin B is secreted predominantly from the Sertoli cells and is involved in negative feedback regulation of FSH; it is also a good indicator of the status of spermatogenesis in adulthood (Anderson and Sharpe, 2000). There is some evidence to suggest that gonadotoxic chemotherapy is associated with a reduction in inhibin B levels in adults (Wallace *et al*, 1997), presumably indicating reduced sperm production (Anderson and Sharpe, 2000). However, this relationship has not been clearly demonstrated in prepubertal boys (Crofton *et al*, 2003). Because spermatogenesis has not yet been initiated, and circulating FSH levels are low, the role of inhibin B before puberty is likely to be rather different than in adulthood (Anderson and Sharpe, 2000). It therefore remains to be seen if inhibin B will become a useful tool in fertility assessment of these children in the future.

1.5 Options for Fertility Preservation

1.5.1 Semen Cryopreservation

Currently the only established option to preserve fertility following gonadotoxic therapy is cryopreservation of spermatozoa prior to commencing treatment. Patients for whom this procedure is suitable must be peri- or post-pubertal and sexually mature. In addition they must be able to give consent for the storage of the specimen.

In the paediatric population, cryopreservation of semen is particularly problematic. Sperm banking has, at least in the recent past, not been universally practiced in Paediatric Oncology centres, and there are few suitable 'adolescent-friendly' facilities. In many

circumstances treatment of the cancer needs to start as quickly as possible following confirmation of the diagnosis and thus obtaining a semen specimen is required relatively soon. After having received such devastating news regarding the diagnosis, it can often be very difficult for teenagers to then discuss fertility and future children and subsequently go on to produce a semen specimen. On the positive side, however, many patients and their families may derive benefit from open discussion regarding fertility, particularly as this places emphasis on looking to the future and provides reassurance that curative treatment is the aim (Wallace and Thomson, 2003).

The semen specimen is usually produced by masturbation but can also be obtained using rectal electrostimulation techniques under anaesthetic. Should it not be possible to obtain an ejaculate, epididymal aspiration or testicular biopsy, in sexually mature men, can be used to retrieve sperm.

The specimens produced are often of poor quality, particularly in the adolescent age group (Postovsky *et al*, 2003). Many of these adolescent patients may have only recently commenced spermatogenesis which may partially explain this, but many other factors are involved including the effects of the disease itself, as discussed above. In addition psychological stress, often observed at this difficult time, can certainly impair semen quality (Clarke *et al*, 1999). The sperm can then sustain further damage as a result of the freeze-thawing process used for cryopreservation, which can impair sperm motility (Alvarez and Storey, 1992) and cause damage to chromatin structure and sperm morphology (Hammadeh *et al*, 1999).

Following cryopreservation, stored spermatozoa are subsequently used to produce offspring via In Vitro Fertilisation (IVF). With advances in assisted reproduction techniques, in particular Intracytoplasmic Sperm Injection (ICSI), which involves the injection of a single spermatozoan directly into an oocyte, the problems of low sperm numbers and poor motility may be circumvented (Chen *et al*, 1996; Rosenlund *et al*, 1998).

Semen cryopreservation should be offered to all suitable patients prior to commencing treatment but, as discussed, this process can be problematic. In addition this option is obviously not applicable to the pre-pubertal patient and thus other methods of fertility preservation must be considered. These alternative options all remain experimental at the present time.

1.5.2 Testicular Tissue Harvesting

As discussed earlier, prepubertal testes do not complete spermatogenesis and thus do not produce mature spermatozoa. However, they do contain the diploid stem germ cells from which haploid spermatozoa will ultimately be derived. Therefore in theory, testicular tissue could be harvested from a biopsy and stored, either as a segment of tissue or as isolated germ cells, prior to sterilizing cancer therapy. Following cure and on entering adulthood this tissue could be thawed and used in one of two ways in order to produce offspring. Firstly, the stored germ cells could be re-implanted into the patient's own testes in order to restore natural fertility, a procedure known as Germ Cell

Transplantation. Alternatively the stored stem cells could be matured *in vitro* until they are able to achieve fertilisation using ICSI.

1.5.2.1 Germ Cell Transplantation

Germ cell transplantation was pioneered in mice by Brinster and colleagues in 1994 (Brinster and Zimmermann, 1994). Following the injection of germ cell suspensions from donor mice into genetically sterile, immune-deficient mice, restoration of spermatogenesis was observed from the donor stem cells. Similar results were also demonstrated in recipient mice that had received sterilising treatment with busulfan. Subsequently, in addition to this heterologous transplantation, successful transfer of germ cells between species has been demonstrated, with rat spermatogenesis noted following transplantation of rat germ cells into the seminiferous tubules of the mouse (Clouthier *et al*, 1996). Germ cell transplantation between phylogenetically more distant species, such as from rabbits and dogs into mice, has not been successful (Dobrinski *et al*, 1999), perhaps because the microenvironment is not suitable for the proliferation of donor spermatogonia. However, spermatogenesis has become established following xenografting of testicular tissue from mice, pigs and goats into castrated, immunodeficient mice (Honaramooz *et al*, 2002).

Whilst the aforementioned studies have been essential in developing germ cell transplantation, the clinical application of this technique in oncological patients would almost certainly require successful autologous germ cell transplantation. Although there have been promising results in primate models (Schlatt *et al*, 1999), beneficial effects

have been difficult to demonstrate because of the inherent difficulty in confirming the origin of subsequent spermatogenesis in these experiments (Schlatt *et al*, 2002).

Although this technique has potential benefit to survivors of childhood cancer, a number of problems first need to be overcome, as discussed later.

1.5.2.2 In vitro Germ Cell Maturation

Maturing germ cells *in vitro*, stimulating their differentiation into spermatozoa, would be particularly useful in patients who have received profoundly gonadotoxic therapy and in whom the supporting Sertoli cells would be unable to support spermatogenesis. Nagano *et al* (1998) demonstrated that mouse spermatogonial stem cells could survive for up to four months in culture, retaining the ability to commence spermatogenesis following transplantation back into a recipient. In addition, Tesarik *et al* (1999) reported the restoration of fertility following *in vitro* spermatogenesis, although this has yet to be confirmed by others. However, this process involved *in vitro* maturation of the later stages of spermatogenesis rather than development from stem cells. Indeed, it appears unlikely that *in vitro* maturation of diploid stem cells into haploid spermatozoa will be technically possible in the near future.

1.5.2.3 Problems with Application of these Techniques

Whilst these techniques, once fully developed, have enormous potential and offer hope to childhood cancer survivors at risk of infertility, these procedures are associated with a number of problems that must be overcome before application in a clinical setting.

Firstly, obtaining the testicular tissue would require an additional surgical procedure following confirmation of the diagnosis and prior to the commencement of any cytotoxic treatment. This would necessitate a further general anaesthetic, although in practice it should be possible to combine the testicular biopsy with other interventions required at this time. In addition, the procedure itself could result in damage to the testis, for example with bleeding and infection of the biopsy site. The prepubertal testis would be particularly vulnerable due to its small size in this age group.

Secondly, autologous germ cell transplantation requires tissue that was removed from a patient with cancer prior to treatment to be returned to the patient following cure. There is, therefore, a genuine risk of reintroducing malignant cells, with potentially fatal consequences. This is unlikely to occur with malignancies such as Hodgkin's disease, which is often localised at presentation, but the risk would be substantial with haematological malignancies (Jahnukainen *et al*, 2001), where the testes can act as sanctuary sites for leukaemic cells. Indeed, any theoretical risk of returning cancer cells following treatment, however small, would not be acceptable.

In order to circumvent this problem, it is likely that the germ cells, and in particular the stem cells, from the testicular biopsy would need to be isolated prior to transplantation. This would be safer and more acceptable than the use of semi-purified cell populations of germ cells and somatic cells as used in transplantation in the rodent models discussed above. However, the term 'stem cell' is a functional description as, at present, there are no specific morphological, antigenic or biochemical criteria to identify these cells.

Although stem cells do express certain surface antigens, such as α -6 and β -1 integrins (Shinohara *et al*, 1999), which would allow purification using magnetic cell sorting (von Schonfeldt *et al*, 1999), other progenitor cells including haemopoietic cells also express these antigens, and thus these markers will not be specific enough to exclude malignant cells. For purification to be effective, specific antibody probes must be developed in order to distinguish germ stem cells from other cells.

A further issue to be resolved before clinical application of these techniques is that of the cryopreservation process. Cryopreservation of mature, haploid spermatozoa is well established. However, there are substantial biological differences between these cells and undifferentiated, diploid stem cell spermatogonia, and thus the requirements of successful freezing and thawing differ considerably. Glycerol is used as the preservative of choice for ejaculated spermatozoa. However earlier germ cells, with proportionately larger amounts of cytoplasm, are more successfully preserved using dimethylsulphoxide (DMSO) as the cryoprotectant (Avarbock *et al*, 1996). Despite this potential, the safety of DMSO in clinical use requires further evaluation prior to human application. Further studies are therefore needed on the optimal cryopreservation process for human testicular stem cells.

For germ cell transplantation to be successful, sufficient numbers of stem cells must be injected into the testes. It has been estimated that 10^4 germ cells isolated from an adult rodent testis contain as few as 2 stem cells (Meistrich and van Beek, 1993). Experiments using murine models have demonstrated that the seminiferous tubules of recipient mice

testes have a volume of approximately 10µl. Since cell concentrations of $1-2 \times 10^8$ cells per ml can be injected, the recipient mouse will only receive approximately 200-400 stem cells in a transplant (Brinster and Nagano, 1998). There is thus a need to develop an *in vitro* enrichment system that will safely augment stem cell numbers following harvesting and isolation, thus improving the cell yield returned to the patient. Nagano *et al* (1998) have demonstrated successful *in vitro* culture of mouse stem germ cells, and thus enrichment is likely to be feasible. However, because of the low numbers of stem cells that are likely to be returned, it may be that patients in whom germ cell transplantation is successful will still only be oligospermic rather than having normal sperm counts. They will therefore still require assisted reproduction in order to produce offspring.

Following harvest, stem cell isolation, cryopreservation and enrichment, the cells then need to be returned to the testes. Although this has been successfully performed in animal models as discussed above, the procedure for transplantation into the human testis remains unclear. The three main routes of re-infusion used previously are multiple microinjections into superficial seminiferous tubules, injection into the rete testis or injection into the efferent ducts. Although all of these techniques have been successful in the mouse (Ogawa *et al*, 1997), the most effective method in larger testes, including humans, is likely to be injection into the rete testis under ultrasound guidance (Schlatt *et al*, 1999). Injection into this area allows a much larger volume to be infused. This technique is particularly successful in immature or regressed testes because of the lower intratubular fluid pressure, which can impair the injection into fully active testes. As the

testes in cancer patients can be regressed secondary to cytotoxic treatment, this technique is certainly promising, although still needs to be refined prior to clinical application.

As can be seen, germ cell transplantation has potential, but there are a number of problems with the technique that need to be resolved. It could be argued that the safest method would be xenografting testicular tissue, for example into nude mice as demonstrated by Honaramooz *et al* (2002). In particular this eliminates the risk of re-introducing malignant cells. There would, however, be a risk of inter-species transfer of potentially pathogenic microorganisms. In addition, and perhaps more importantly, this process would raise ethical difficulties, which may limit its clinical application.

The technique of *in vitro* maturation of stem cells shares many of the problems discussed above but also circumvents the risk of re-introducing malignant cells, thus making this procedure potentially highly beneficial in this patient group. However, as discussed above, the technology to enable this maturation to occur may be some time away.

Although ICSI has been successful using nuclear material from a round spermatid (Tesarik *et al*, 1995) and thus the maturation to spermatozoa need not be complete, the ability to artificially mature a diploid stem cell into a suitable haploid cell is not possible at the present time.

Finally, ICSI itself is not without potential complications. Because ICSI involves the injection of nuclear material directly into an oocyte, some of the natural mechanisms that normally prevent sperm with defective DNA being involved in fertilisation are bypassed.

In addition, the oocyte fertilized may itself be abnormal. There is, therefore, genuine concern that DNA and chromosomal abnormalities are more likely to be passed on to offspring produced with such techniques. Indeed, a number of large follow up studies have demonstrated an increase in chromosomal abnormalities, particularly of the sex chromosomes, in offspring born using ICSI (Tarlatzis and Bili, 2000; Bonduelle *et al*, 2002a). However, rather than the technique itself predisposing to these abnormalities, the increased incidence is felt to reflect the higher rate of constitutional chromosomal anomalies observed in infertile couples utilizing ICSI, particularly on the paternal side (Bonduelle *et al*, 2002a).

Transmission of constitutional chromosomal anomalies is less likely in cancer survivors because their infertility is secondary to gonadotoxic treatment. However, concerns have been raised that the mutagenic potential of cancer therapy could predispose the offspring of these patients to congenital abnormalities, and even cancer themselves. A large epidemiological study has failed to demonstrate any such link (Hawkins *et al*, 1989), except in those with familial malignancies, although these offspring resulted from natural conception. A recent study (Thomson *et al*, 2002a) investigated the integrity of spermatozoal DNA in men who had undergone treatment for childhood cancer. These sperm did not carry a greater burden of damaged DNA as compared to age-matched controls, providing some reassurance with regards to the use of spermatozoa from oligospermic men who have survived childhood cancer.

A further concern of offspring born using ICSI and IVF is the risk of congenital malformations. A number of large follow up studies have demonstrated no difference between ICSI and IVF in this regard, and indeed have suggested a comparable rate of major malformations to that seen following natural conception (Bonduelle *et al*, 2002b; Palermo *et al*, 2000). Other studies have suggested an increased malformation rate, although have attributed this to the higher rate of multiple births seen following assisted reproduction (Wennerholm *et al*, 2000).

However, interpretation of studies such as these can be problematic due to the inherent difficulty in obtaining appropriate control data for comparison, as maternal age, parity, sex of the infant and multiple births will affect the incidence of these abnormalities. In addition, variability exists between definitions of major and minor birth defects. In contrast to the aforementioned studies, Hansen *et al* (2002) suggested that infants conceived using ICSI or IVF are twice as likely to suffer a major birth defect, and that this risk remained significant even allowing for these factors. Infants born of such techniques will be followed up more closely and thus one could argue that defects are more likely to be diagnosed in this cohort. However, the authors included defects diagnosed up to one year of age, at which time the majority of major defects would have been detected in all patient groups. In addition, there is evidence to suggest that babies born following assisted reproduction are at greater risk of low and very low birth weight (Schieve *et al*, 2002). Although this can again be explained, in part, by an increase in multiple births, it can also be a feature of singletons born with this technology.

Further long-term prospective studies are required to obtain accurate information on these children. If an increased risk of abnormalities is genuine, consideration must be given to the aetiology of these problems. The difficulty in identifying causative factors is that couples utilizing these techniques are inherently more at risk prior to fertility treatment, and thus the effect of the procedure itself can be difficult to elucidate. As discussed above, these risk factors may be less likely in survivors of childhood cancer.

In summary, both germ cell transplantation and *in vitro* maturation do offer hope for fertility preservation in males treated for cancer, particularly those in the prepubertal age group. However, in view of the significant concerns that exist with both techniques, it may be some time before their clinical potential is realized and they form part of the routine management in these patients.

1.5.3 Hormonal Manipulation

Cytotoxic treatment, as mentioned previously, acts principally on rapidly dividing cells. As the testis demonstrates much cellular activity it is therefore prone to this damage and gonadotoxicity may result. In view of this, it has been postulated that inducing testicular quiescence could prevent gonadal toxicity, by making germ cells less vulnerable to the cytotoxic effects. This may represent an alternative, and potentially more acceptable method of fertility protection in prepubertal boys treated for cancer.

1.5.3.1 Rodent Models

Studies investigating this technique using rodent models have produced very encouraging results. Ward *et al* (1990) induced suppression of the hypothalamic-pituitary-gonadal axis using a GnRH agonist. Pulsatile GnRH is required for normal gonadal function, but if administered at a supraphysiological dose in a non-pulsatile, chronic manner, suppression of the hypothalamic-pituitary-gonadal axis will occur via down-regulation of pituitary GnRH receptors. When administered to rats prior to treatment with procarbazine, enhanced recovery of spermatogenesis was demonstrated, as compared to control animals. Similar protection of spermatogenesis has also been demonstrated using other methods of suppressing the reproductive axis, including treatment with testosterone, testosterone and oestradiol (Parchuri *et al*, 1993), GnRH antagonists and anti-androgens such as flutamide (Kangasniemi *et al*, 1995). In addition, these methods have been protective against other gonadotoxic agents such as cyclophosphamide (Meistrich *et al*, 1995) and radiotherapy (Kurdoglu *et al*, 1994), suggesting that the mechanism of protection is not specific to particular modes of cytotoxic therapy.

A number of issues have arisen from these experiments. Firstly, the time required to suppress the reproductive axis is an important factor if this procedure is to be utilized in a clinical setting. Many of the studies discussed above involve hormonal manipulation commencing a number of weeks prior to administration of the cytotoxic agent. Cancer treatment should, in general, start as soon as possible following confirmation of the diagnosis, and therefore this delay would not be acceptable in patient management.

Secondly, the timing of suppression of the reproductive axis in relation to the cytotoxic damage has provoked many questions regarding the mechanism of gonadal protection. Meistrich and Kangasniemi (1997) demonstrated that hormonal manipulation with a GnRH antagonist or testosterone led to the successful recovery of spermatogenesis in rats even when given *after* irradiation. Similar results have also been observed with hormonal treatment after the administration of procarbazine (Meistrich *et al*, 1999). Whilst this would circumvent the concerns highlighted above regarding the delay in cancer treatment, these studies suggest that the original hypothesis of gonadal protection simply involving a reduction in the susceptibility of germ cells is incorrect.

There is evidence, based on rodent studies, to suggest that azoospermia following cytotoxic therapy does not necessarily result from complete depletion of spermatogonia, but can be secondary to a failure of surviving spermatogonia to replicate and differentiate (Kangasniemi *et al*, 1996). It has therefore been postulated that the recovery of spermatogenesis following hormonal treatment observed in these rodent models is due to stimulation of differentiation of surviving spermatogonia. A consistent feature observed in recovery of spermatogenesis is a reduction in intratesticular testosterone (Meistrich and Kangasniemi, 1997; Shetty *et al*, 2000), and it has been suggested that this relieves the block of differentiation, perhaps by alterations in the endocrine and paracrine environment of the testes. This subsequently allows the restoration of spermatogenesis.

1.5.3.2 Human and Primate Studies

Despite the success of these techniques in rodent models, clinical trials of hormonal manipulation in male patients receiving gonadotoxic therapy have thus far failed to demonstrate any benefit. Administration of a GnRH analogue prior to and during treatment for lymphoma has been ineffective in conserving fertility (Johnson *et al*, 1985; Waxman *et al*, 1987). Interestingly, the use of GnRH analogues to suppress the hypothalamic-pituitary-ovarian axis in both female patients and female primate models has demonstrated gonadal protection from alkylating agents (Ataya *et al*, 1995; Blumenfeld *et al*, 1996).

Subsequent to the earlier trials of male patients, and in view of the studies discussed above regarding the optimal timing of hormonal treatment, Thomson *et al* attempted to restore spermatogenesis in seven men rendered azoospermic following treatment for childhood cancer (Thomson *et al*, 2002b). However, after suppression of the hypothalamic-pituitary-gonadal axis with testosterone and medroxyprogesterone acetate the men remained azoospermic on reassessment, and their testes, on biopsy, lacked germ cells. Perhaps in this study the hormonal treatment was initiated too long after the cytotoxic therapy. In addition, the lack of success may be because the initial testicular insult was too severe, and that this technique may be more beneficial in patients with less severe gonadal damage in whom some spermatogonial stem cells are preserved following cytotoxic treatment. However, it is difficult to justify further clinical trials in this area until the mechanism of gonadal 'protection' is better understood.

Indeed, extrapolating evidence from rodent models to clinical trials assumes that both the physiology of spermatogenesis and the mechanisms of gonadotoxic damage are similar in both species. However, as discussed previously, there is evidence to suggest that significant inter-species differences do exist, and that perhaps other primates would be more appropriate models in which to study spermatogenesis in this regard (Weinbauer and Nieschlag, 1989; Sharpe *et al*, 2000). Certainly, hormonal manipulation in the macaque does not prevent radiotherapy-induced gonadal damage (Kamischke *et al*, 2003).

1.5.3.3 The Marmoset as a Model for studying Testicular Development

The common marmoset (*Callithrix jacchus*), a New World primate (Fig 1.7), represents a useful model for studying prepubertal testicular development.

Firstly, the marmoset demonstrates similar phases of testicular development as the human (Sharpe *et al*, 2000). The testes are descended into the scrotum at birth, or within days of birth. During the neonatal period the marmoset demonstrates a surge of testosterone (Fig 1.8), as occurs in the human. As discussed previously, the function of this hormonal activity is uncertain at present. Following this, the hypothalamic-pituitary-gonadal axis becomes relatively quiescent during a relatively prolonged prepubertal phase (Fig 1.8). The marmoset then commences puberty at around 45 weeks of age, with a concomitant increase in hormonal activity, subsequently entering adulthood at around 75 weeks of age.

Figure 1.7 Adult Marmoset, *Callithrix jacchus*

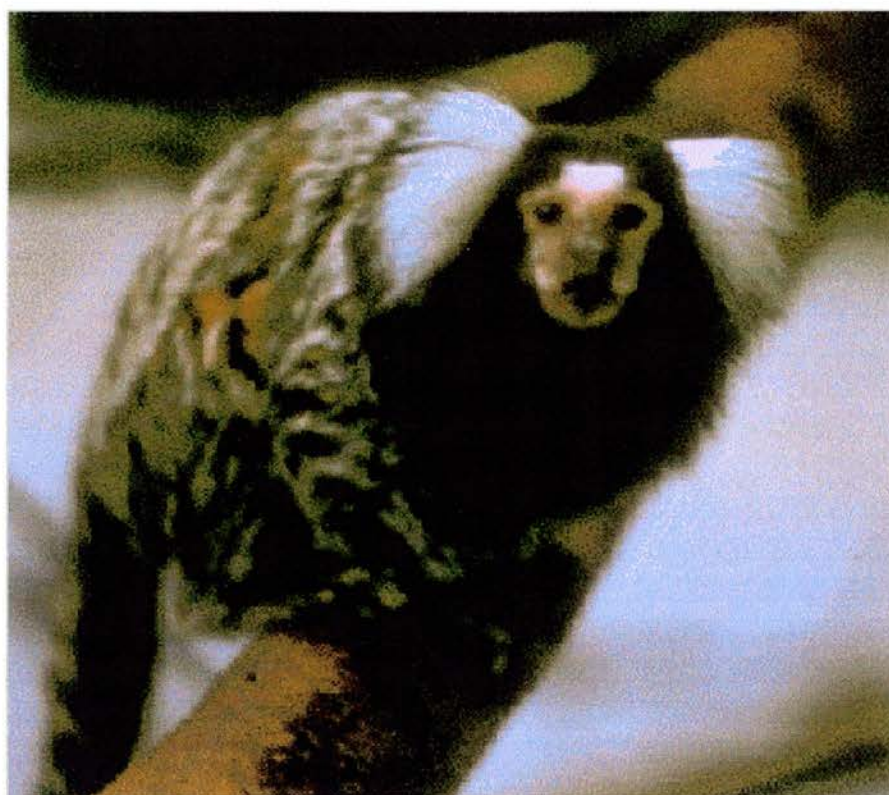
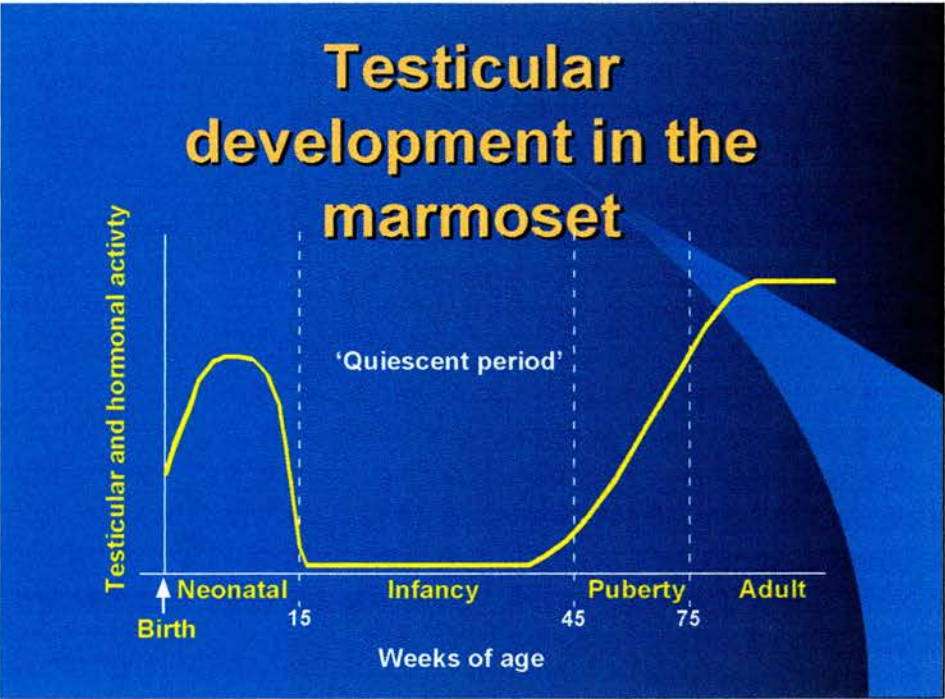


Figure 1.8 Hormonal Activity during Testicular Development in the Marmoset



Secondly, the marmoset displays similarities in its organization of spermatogenesis to the human (Miller *et al*, 2000). These similarities relate both to the stages and duration of the spermatogenic cycle, and to the arrangement of these stages along the seminiferous tubule. Of those primates studied, spermatogenesis in the marmoset most closely resembles that in the human and chimpanzee, and is certainly more comparable than spermatogenesis in the rat. It is also established that spermatogenesis in the marmoset is of similar poor efficiency to that observed in the human (Sharpe *et al*, 2000).

A further advantage of using the marmoset as an animal model is that they are usually born as twins, which are dizygotic but share a common placenta. Therefore any intervention in one animal can be compared with a genetically distinct, but otherwise similar control animal. This reduces the number of animals required to achieve statistically significant results, and is particularly important as considerable inter-animal variability can occur.

1.5.3.4 Prepubertal Testicular Development in the Marmoset

Using this marmoset model, Kelnar *et al* (2002) investigated the prepubertal phase of testicular development. This study was based on the immunoexpression of a number of antigens within prepubertal marmoset testes.

Functional development of Sertoli cells, based on the expression of sulphated glycoprotein-2 and androgen receptor, and of Leydig cell activity, based on the expression of 3 β -hydroxysteroid dehydrogenase, was demonstrated prepubertally. In



addition, proliferation of germ cells was noted at this age, indicated by the immunoexpression of proliferating cell nuclear antigen (PCNA). This provides further evidence that the prepubertal testis is not quiescent, and improves our understanding of why the testis is susceptible to cytotoxic damage at this age group.

In addition, this study also investigated the effects of treatment with a GnRH antagonist on the prepubertal marmoset. It was found that this treatment, for ten weeks during the prepubertal phase between 25 and 35 weeks of age, largely prevented many of the changes highlighted above. However, quite unexpectedly, the proliferation of germ cells, as assessed by the PCNA labeling index of spermatogonia, was unaffected by administration of the GnRH antagonist, when compared to control animals (Kelnar *et al*, 2002). This suggests that spermatogonial, and possibly stem cell, proliferation, at least in prepubertal primates, is in fact gonadotrophin-*independent*.

If this finding were to be confirmed it would suggest that hormonal manipulation based on suppression of the gonadotrophin axis is unlikely to be successful in alleviating gonadotoxic damage secondary to cancer treatment, and thus may explain the failure of previous studies using this technique in primates.

This project aimed to investigate further the hypothesis that prepubertal spermatogonial proliferation in primates is gonadotrophin independent, using alternative proliferative markers. Assuming that this was confirmed, the intention was then to investigate what

factors do regulate spermatogonial proliferation at this age, with the hope that these would offer novel targets for gonadal protection during cytotoxic therapy.

Chapter 2

Immunohistochemistry- Basic Principles and Techniques Used

2.1 Introduction

The basis of this study is the immunohistochemical detection of proteins present in fixed marmoset testis tissue. Immunohistochemistry was selected as the most appropriate method of investigation as it not only establishes the presence of certain proteins in a tissue, but also allows the localisation of the protein within that tissue. Indeed, in order to elucidate what factors act on a subtype of cells (spermatogonia and stem cells) within a multicellular tissue (testis), immunohistochemistry is the only viable approach. In addition, many methods of investigation that have contributed to our understanding of spermatogenesis in rodents, such as gene knockout models, are not applicable to primate species.

The essential factor common to all immunohistochemical techniques is the primary antibody. Specific antibodies to increasing numbers of tissue antigens are now available, which has contributed both to the quantity and quality of immunohistochemical techniques now possible. In addition to the antibodies themselves, refinements to many areas of the process have enabled immunohistochemistry to have widespread application, both in research and as a diagnostic tool in cellular pathology. For example, the use of enzyme labelling has enabled cellular morphology to be viewed in conjunction with antibody localisation. In addition, the use of antigen retrieval methods have led to the detection of antigens not previously possible in sections fixed in formalin.

However, despite these improvements it is important to note that the lack of immunostaining is not proof of absence of the antigen, as the antigen may be present but not detectable with the method used. Immunohistochemical methods require to be optimised for individual tissues, with the ability to detect the presence of an antigen balanced against the conservation of cellular morphology.

This chapter is not intended as a comprehensive overview of immunohistochemistry, but rather to focus on areas relevant to the present study. The methods discussed relate to those used for the investigation of antigens within the marmoset testis, including the use of immunofluorescence and alternative methods of double staining. Background (non-specific) staining is also discussed, as this was found to be the major problem with the immunohistochemical analysis of the marmoset tissue studied.

2.2 Principles of Immunohistochemistry

Immunohistochemistry is a technique for identifying cellular or tissue constituents (antigens) by means of their specific binding to an antibody. The site of this binding can then be detected, either by direct labelling of the antibody, or by use of a secondary labelling method.

2.2.1 Antibodies

IgG is the most common subclass of primary antibody used in immunohistochemistry, and is produced in both polyclonal and monoclonal forms. Polyclonal antibodies are produced by immunising an animal with a specific molecule bearing the antigen of interest (De Mey and Moeremans, 1986). An immune response is generated, and the

antibodies produced can be harvested from the animal's blood. Because numerous clones of plasma cells are produced, the antibodies have slightly different specificity, reacting to various epitopes on the antigen against which they are raised. In contrast, monoclonal antibodies are the product of an individual clone of plasma cells and are therefore identical, reacting to a specific epitope against which they are raised (Gatter *et al* 1984). After an immune response has been generated, B-lymphocytes from spleen or lymph nodes are harvested and fused with non-secreting mouse myeloma cells. This hybrid results in the production of specific antibody from an immortal cell line. Unlimited quantities of antibody, which is both pure and highly specific, can therefore be obtained.

Monoclonal antibodies demonstrate high homogeneity, with little variability between batches. Consistent results are therefore reliably obtained with antibodies produced in this manner. However, the conditions for the use of monoclonal antibodies are highly specific, and must be identical to those used in quality control on production of the antibody initially. In addition, polyclonal antibodies may still detect antigens that are not adequately preserved by tissue fixation, whereas the particular epitope with which the monoclonal antibody interacts may not be preserved.

2.2.2 Antibody Binding

The affinity of antibody binding to the antigen is determined, to a large extent, by the specificity of the antibody used. Antigen-antibody reactions are reversible and thus immune complexes may dissociate during the washing cycles used in immunohistochemistry. Antibodies with higher affinity are advantageous in this regard, as this is less likely to occur.

Under ideal conditions, antibodies react with their antigen very rapidly. However, in immunohistochemistry, conditions such as tissue fixation, antibody concentration and temperature affect the rate of this reaction. Because equilibrium between antigen-bound and free antibody is rarely achieved, long incubation periods with more dilute primary antibody are generally used. The use of more concentrated antibody preparations frequently results in non-specific background staining, affecting the interpretation of results. When investigating the immunoexpression of an antigen within a particular tissue using a particular primary antibody, it is therefore essential to initially use a range of antibody concentrations, in order to identify the conditions for optimal staining.

2.2.3 Labelling

In order for the site of antigen-antibody reaction to be visualised, the antibody used must be labelled. Enzymes are the most widely used labels in immunohistochemistry, and incubation with a chromogen produces a stable, coloured reaction end product, suitable for visualisation under the light microscope.

Horseradish peroxidase is the enzyme most frequently used and, in combination with 3,3-diaminobenzidine tetrahydrochloride (DAB), yields a stable, dark brown reaction end product (Graham and Karnowsky, 1966). However, endogenous peroxidase activity within the tissue, present in a number of sites such as neutrophils and other myeloid cells, must be blocked before application of the primary antibody. Hydrogen

peroxide in combination with methanol (Streefkerk, 1972) is used as the blocking procedure in this study.

2.2.4 Staining methods

The simplest method of staining is the direct technique, in which the primary antibody is enzyme-labelled, and this reacts directly with the antigen in the tissue (Fig 2.1).

This method is quick and non-specific reactions are limited. However, because signal amplification cannot be achieved, the sensitivity of this method is insufficient for most immunohistochemical studies.

Sensitivity can be improved with the use of an indirect, two-step method. Here, the primary antibody binds to the antigen within the tissue as before. However, the enzyme label is not attached to the primary antibody but to a secondary antibody, which itself binds to the primary antibody (Fig 2.2). This secondary antibody must be directed against immunoglobulins from the same species in which the primary antibody was raised. Several secondary antibodies are likely to react with a number of different epitopes of the primary antibody, and therefore more enzyme molecules will be attached to each target site, thus amplifying the signal obtained. Undesired reactions may occur if the secondary antibody cross-reacts with immunoglobulins present within the tissue specimen. However, the use of a secondary antiserum that has been preabsorbed with immunoglobulins from the species under investigation can eliminate this cross-reactivity.

Figure 2.1 Direct Method

Enzyme-labelled primary antibody reacts with tissue antigen

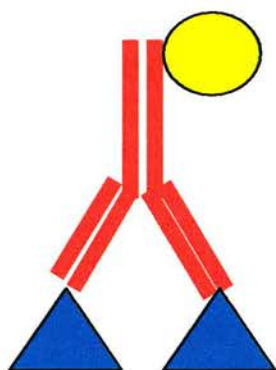


Figure 2.2 Two-step indirect method

Enzyme-labelled secondary antibody reacts with primary antibody bound to tissue antigen

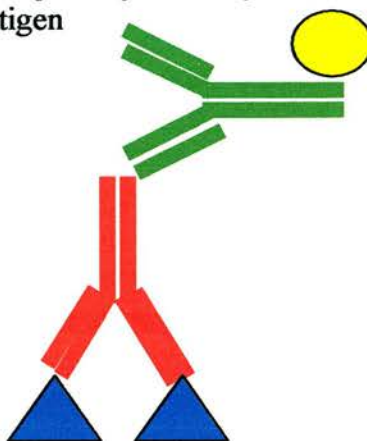


Figure 2.3 Avidin-Biotin Technology

Avidin- or streptavidin-biotin enzyme complex reacts with the biotinylated secondary antibody

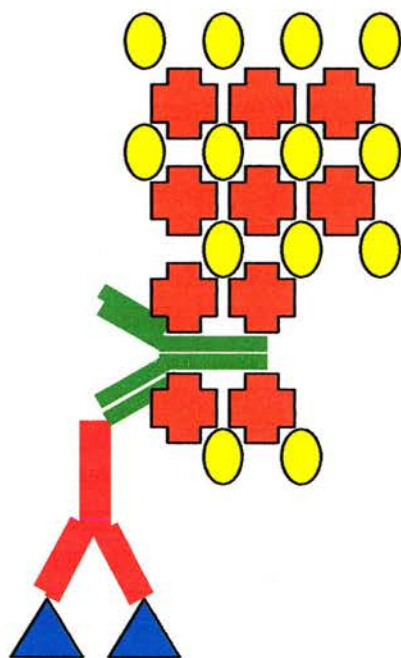
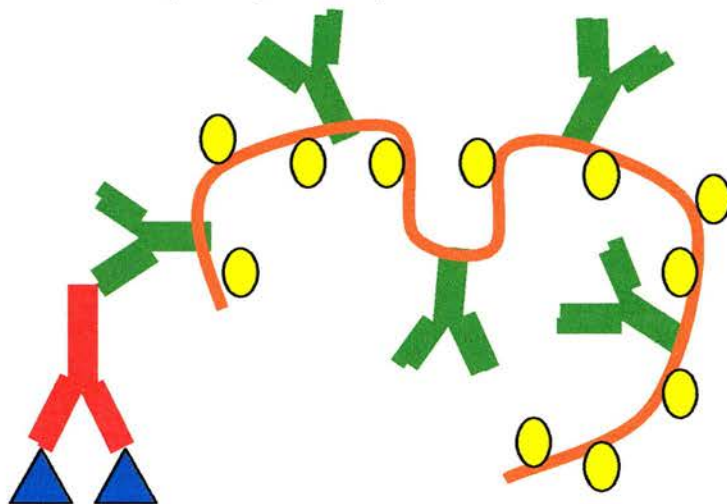


Figure 2.4 Chain polymer-conjugated Technology (Envision)

A spine molecule, which contains an average of 10 molecules of secondary antibody and 70 molecules of enzyme, binds to primary antibody



Key to figures

Primary Antibody



Tissue antigen



Enzyme



Secondary antibody



Avidin-biotin complex



If required, further amplification can be obtained by the addition of an enzyme-labelled tertiary antibody to the complex. This three-step indirect method can be useful for staining antigens with a limited number of epitopes.

Many immunohistochemical staining methods now use avidin-biotin techniques, which rely on the high affinity of the glycoprotein avidin, or streptavidin, for biotin, a low molecular weight vitamin. The principle is identical to the two-step indirect method, except the secondary antibody is not enzyme-labelled but is biotinylated. The enzyme label is attached to an avidin-biotin complex, and this binds to the biotin on the secondary antibody (Fig 2.3). This signal amplification results in increased sensitivity of detection (Hsu *et al*, 1981). The main problem with this technique is non-specific staining from endogenous biotin, which can affect the interpretation of results.

More recently, systems using chain polymer-conjugated technology have been developed. An inert dextran molecule acts as a spine, onto which both enzyme labels and secondary antibody can be attached (Fig 2.4). On average, 70 molecules of enzyme and 10 molecules of antibody can be attached to the spine, allowing even greater sensitivity of detection. In addition, the problem of non-specific binding of endogenous biotin is eliminated. This technology is now commercially available, and the system used in this study is Envision (DAKO, California, USA). However, at present this system is only available for the detection of primary antibodies raised in either rabbit or mouse, and therefore the avidin-biotin method is required for antibodies raised in other species, such as the goat.

2.3 Tissue Fixation

To enable reliable detection of antigens within a tissue, it is essential that these antigens are adequately preserved. In addition, conservation of cellular morphology is required to allow the precise cellular and sub-cellular localisation of those antigens detected. Fixation achieves this by stabilizing proteins within the tissue, by the formation of cross-links, and the methods employed depend on both the tissue itself and the investigative technique to be used.

Although this cross-linking acts to stabilize proteins, it can also result in the ‘trapping’ of antigens, such that antibodies cannot physically access them in order to bind. The longer and stronger the fixation, the more widespread is the cross-linking. Antigens affected in this manner can be detected following antigen retrieval procedures, as discussed in the following section, but tissue morphology can be impaired following such techniques. This emphasises how immunohistochemical methods must be optimised to ensure both adequate antigen detection and cellular morphology. Frozen tissue avoids the problems of cross-linking, but as a consequence morphological preservation is drastically impaired. The use of frozen tissue would therefore not be suitable for the present studies, in which accurate localisation and identification of specific sub-sets of spermatogonia is a prerequisite.

The marmoset testes used in this study were fixed in Bouin’s solution for 5-6 hours, and then embedded in paraffin. Bouin’s solution is formaldehyde based, and achieves fixation by reacting with basic amino acids to form cross-linking ‘methylene bridges’. This results in relatively low permeability to macromolecules, and means that structures of intracytoplasmic proteins are not significantly altered (Fox *et al*, 1985).

Although, as mentioned, some antigens may not be well demonstrated after fixation in formaldehyde-based fixatives, these may be detected following antigen retrieval methods, particularly if polyclonal antibodies are used.

2.4 Antigen Retrieval

Because the immunoreactivity of certain antigens can be affected by tissue fixation, particularly by the use of formalin, numerous techniques have been developed to improve the detection of these antigens, a process known as antigen retrieval.

Antigen retrieval has been reported following proteolytic digestion (Huang *et al*, 1976) and microwave heating (Shi *et al*, 1991). However, the study of marmoset testis detailed here uses pressure-cooking as a heat-mediated antigen retrieval step, as described by Norton *et al* (1994), because heating of the tissue is more uniform than with microwave heating. In addition, more slides can be heated in one batch. This heat treatment does not appear to significantly alter cellular morphology of fixed tissue (McNicol and Richmond, 1998), although some loss of cellular architecture will occur and therefore conditions must be optimised to minimize this disruption.

For antigen retrieval to be effective, the slides must be heated in an aqueous solution. The solution used in the present study was most commonly a citrate buffer with a pH of 6.0, although a glycine buffer with a pH of 3.5 was also used on occasions, and alternative buffers can be effective. Optimising the method of retrieval for a particular antigen in a particular tissue requires the use of different buffers, in order to assess which is most appropriate.

The degree to which immunoreactivity can be restored is also related to the duration of heating and the temperature attained. Sections in the present study were kept at boiling point for 5 minutes, before being allowed to cool slowly over 20 minutes, as this has been previously shown to be effective using this marmoset tissue (Kelnar *et al*, 2002).

Whilst the exact mechanism of action of antigen retrieval remains uncertain, it is felt that these methods may be effective by breaking down some of the protein-protein cross links induced by tissue fixation.

It is important to note that not all antigens require antigen retrieval before their detection. In general, detection of antigens within the nucleus is improved with antigen retrieval, whereas cytoplasmic antigens can often be detected without this step.

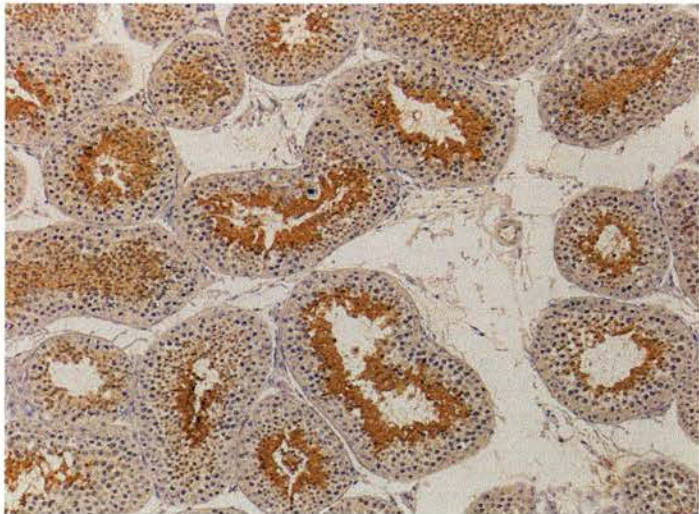
2.5 Background Staining

Background staining is one of the most common problems encountered in immunohistochemistry, and certainly caused difficulties in this study of the marmoset testis (Fig 2.5).

Background staining may be specific, such as fibrinogen in blood vessels, or, as demonstrated in Fig 2.5a, staining of elongate spermatid cytoplasm within post-pubertal sections of marmoset testis, which can be a problem at this age. Another common site of background staining in this tissue is the acrosome of spermatids.

Figure 2.5 Examples of Background Staining observed in Marmoset Testis

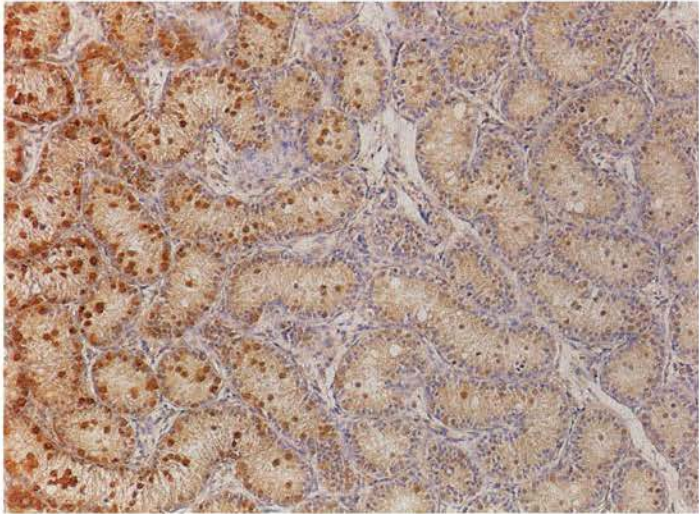
(a) Section from adult marmoset demonstrating staining of cytoplasm of elongate spermatids (10x magnification)



(b) Section from prepubertal marmoset demonstrating non-specific staining within the tissue (20x magnification)



(c) Variable tissue fixation resulting in inconsistent immunostaining (10x magnification)



Alternatively, background staining may be non-specific (Fig 2.5b), due to the apparent affinity of certain tissue components. Non-specific staining is usually the most problematic source, and is caused mainly by non-immunological binding of antibody to certain sites within tissue sections via hydrophobic and electrostatic sources (Kraehenbuhl and Jamieson, 1974). This binding can be of the primary antibody or, more commonly, the secondary antibody, which can be particularly problematic due to the considerable variability between batches. By blocking these sites that have a non-specific affinity for antibodies, this background staining can be reduced. This can be achieved by incubating the sections in immunoglobulin that will bind to these sites but will not react with the primary antibody. Normal (diluted) whole serum from the species in which the secondary antibody is raised is therefore applied to the sections before application of the primary antibody. In addition, the primary antibody itself is diluted in this normal whole serum. Lower concentrations of primary antibody will also reduce the background, and with the more sensitive staining methods discussed above, such as Envision, detection of genuine staining is still possible. Further methods to reduce background staining include the addition of a detergent such as Tween into the washes, in order to reduce the hydrophobic interactions.

A further source of background staining can be from endogenous enzymes. As mentioned previously, endogenous peroxidase activity must be blocked before application of the primary antibody, and this is usually achieved by incubation in methanol and hydrogen peroxide. Biotin-based detection methods are susceptible to background staining from endogenous biotin. Biotin is present in a variety of tissues, including liver, kidney and lymphoid tissue, but is also likely to contribute to the

background staining observed within the lumen of the seminiferous tubule of the marmoset testis. Incubation of sections with avidin, followed by biotin, prior to application of the primary antibody, reduces the background from this source (Wood and Warnke, 1981).

Finally, background staining can be secondary to tissue damage, either by drying out prior to fixation or by incomplete penetration of the fixative into the tissue (Fig 2.5c). This generally appears as diffuse staining of all or most tissue elements within the affected area.

2.6 The Use of Controls

The use of controls in immunohistochemistry is essential for validation of the results, particularly with regards to the specificity of the primary antibody. False-positive results can certainly occur with the use of polyclonal antibodies, because related molecules may have antigenic components in common. Although this is less likely with the use of monoclonal antibodies, non-specific cross-reactivity can still occur.

For immunohistochemical staining to be specific it must be shown that staining does not occur in the absence of the primary antibody. Furthermore, staining should be inhibited by adsorption of the primary antibody with the relevant antigen prior to its use.

In practice, the following types of controls are routinely used in immunohistochemistry. Firstly, negative controls involve a section treated identically to all other sections, except for the omission of primary antibody. Any staining

present on this tissue would therefore originate from an alternative source, such as the secondary antibody, and thus interpretation of other sections investigated would be problematic. Secondly, a blocking peptide can be used, in which the primary antibody is preabsorbed with purified antigen, before application to the tissue. Similarly, no staining should be observed on this section; this is probably the most convincing control.

Finally, a positive control is advisable, as the absence of staining in a section does not necessarily imply that the antigen is not present. Using tissue from the same animal that is known to express the antigen under investigation will confirm that detection with the primary antibody can be demonstrated. Selection of an appropriate tissue as a positive control, however, can be problematic for some antigens, for example those expressed mainly by 'stem cells'.

2.7 Double Staining

Double staining, as its name suggests, allows the immunohistochemical detection of two antigens in the same section of tissue. Prior to the development of double staining techniques, the analysis of serial sections was required. This process is both laborious and co-expression can be difficult to visualise. In contrast, co-localisation enables direct visualisation of the expression of multiple antigens in the same tissue section.

If the two antigens are present within the same cellular compartment, co-localisation will be demonstrated by the presence of a mixed colour. When the two antigens are expressed in different cellular compartments, or in separate cells of the section, two

separate colours will be observed. These colours should therefore be of sufficient contrast to demonstrate this.

The double staining methods predominantly used in this study are based on immunofluorescence, with analysis using a confocal microscope. However, double-staining methods based on conventional immunoenzymatic techniques similar to those discussed above can also be used.

2.7.1 Immunofluorescence

The basic principles of immunofluorescence, with specific antibody binding to an antigen within the tissue, are identical to conventional immunohistochemistry discussed above. However, instead of an enzyme-labelled secondary antibody that produces a coloured end product with a chromogen, the antibodies are labelled with a fluorochrome, which has the property of absorbing radiation in the form of ultraviolet or visible light. This absorbed radiation causes the molecule to attain an 'excited state', leading to electron redistribution and the emission of radiation of a different wavelength. The emitted light is almost invariably of a longer wavelength and within the spectrum detectable by the confocal microscope.

By using fluorochromes that emit light at different wavelengths, different antibodies binding to different antigens can be visualised as separate colours, usually red, green or blue. This produces excellent contrast when the antigens are expressed separately, and allows simple visualisation if the antigens are expressed within the same cellular compartment.

The main drawback of analysing sections using immunofluorescence is the difficulty visualising the cellular morphology. Although fluorescent counterstaining can assist interpretation, the information obtained may be limited when compared to conventional staining using light microscopy.

The sensitivity of detection can also be problematic using immunofluorescence. The fluorescent signal can be amplified using enhancement with tyramide, as used in this study. This is an enzyme-mediated detection method utilising horseradish peroxidase to generate high-density labelling of a target protein (Bobrow *et al*, 1989). However, although sensitivity of detection is increased, background staining also becomes more prominent, particularly autofluorescence caused by formaldehyde fixation.

Finally, sections stained using immunofluorescence should be kept in cool temperatures of around 4°C to prevent fading of the signal with time. In addition, they should be protected from direct light, both during the staining procedure and for subsequent storage.

2.7.2 Alternative Double Staining Methods

Double staining using conventional immunohistochemical methods can also be achieved. As mentioned previously, incubation of horseradish peroxidase with DAB produces a dark brown colour. However, enzyme labels on different antibodies applied to the same section can be combined with alternative chromogens to produce contrasting colours. Chromogens used in this study include Fast Blue, which produces a blue colour, and NBT, which stains purple.

It can be more difficult to visualise co-expression using these techniques, as compared to immunofluorescence. However, this procedure does enable easier interpretation of the staining pattern in context with the cellular morphology. In addition, if immunopositive cells are infrequent within a section, such as stem cells within the testis as investigated in this study, these double-staining procedures can be advantageous.

Chapter 3

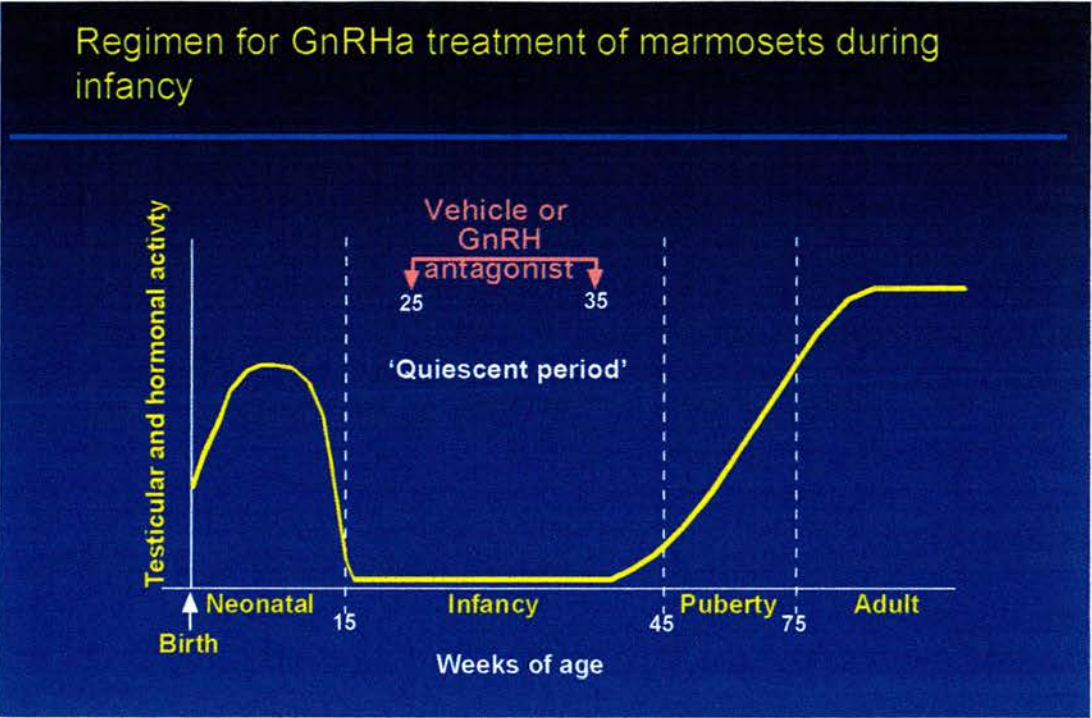
Materials and Methods

3.1 Animals and treatments

Animals were captive-bred common marmoset monkeys (*Callithrix jacchus*), maintained in a colony that has been self-sustaining since 1973. A total of 16 infant male marmosets aged 25 weeks of age were treated weekly with either vehicle (controls; $n=7$), or with 10mg/kg of a potent long-acting Gonadotrophin Releasing Hormone (GnRH) antagonist (Antarelix; Europeptides, Argentueil, France) ($n=9$) until 35 weeks of age. Treatments were administered as weekly subcutaneous injections in propylene glycol: water (1:1, v:v). As demonstrated in Figure 3.1, this treatment is administered during the prepubertal ‘infancy’ phase of testicular development in the marmoset. Although gonadotrophin levels cannot be measured in this species, and thus the effectiveness of GnRH antagonist treatment cannot be directly assessed, this treatment has previously been shown to be effective. Complete suppression of the neonatal testosterone surge has been achieved with this agent, as evidenced by testosterone assays in this species (Lunn et al, 1994, 1997). In addition, Sertoli and Leydig cell development in this species is impaired by such treatment (Sharpe *et al*, 2000; Kelnar *et al*, 2002).

Because marmosets demonstrate considerable inter-animal variability that would normally necessitate the use of large numbers of animals in experiments, the present study, where possible, used male co-twins ($n=4$), which tend to be highly comparable. Therefore, whilst one co-twin was administered the GnRH antagonist, the other co-twin received vehicle as a control. This design enabled pair-wise comparison of data

Figure 3.1 The timing of administration of the GnRH antagonist in relation to normal testicular development in the marmoset



for each control and treated co-twin at 35 weeks of age, thus minimizing the number of animals required for study.

To increase our understanding of germ cell proliferation at all stages of testicular development, testes from animals killed at various time points, ranging from the neonatal period through to adulthood, were also included in the study. These time points were as follows: age 1-6 days ($n=10$); 4 weeks ($n=4$); 6 weeks ($n=5$); 14 weeks ($n=3$); 18-24 weeks ($n=5$); 58-62 weeks ($n=4$) and adulthood ($n=4$). The variation in numbers within each age group reflects the availability of animals at these time points.

As discussed previously, the role of the neonatal testosterone surge, observed in a number of primates including both man and marmoset, is uncertain. In order to establish whether this hormonal activity may have a role in the regulation of spermatogonial proliferation, this study also included analysis of testes from 4-week-old animals in which the neonatal testosterone surge had been suppressed by prior administration of a GnRH antagonist. Analysis of co-twin pairs ($n=4$) was used as above, with one co-twin administered the GnRH antagonist on the day of birth, day 3, day 7 and then at weekly intervals until week 4, at which time the animal was killed. The other co-twin received vehicle as a control, at the same time intervals.

These studies were approved by the local ethics committee for studies in primates and were performed according to the Animal Scientific Procedures (UK) Act (1986) under Project Licence approval by the UK Home Office. The author is grateful for the assistance of Dr R Sharpe in the administration of treatments to the animals involved.

3.2 Tissue collection and processing

Animals were killed by injection of an overdose of sodium pentobarbitone (Euthatal; Rhone Merieux Ltd, Harlow, Essex, UK). Testes with epididymides attached were dissected free of connective tissue and immersion-fixed for 5.5 hours in Bouins, after which the testis was dissected away from the epididymis and weighed. Fixed testes were then processed overnight in an automatic processor and embedded in paraffin. Testicular sections of 5µm thickness were then mounted on charged glass slides (BDH Chemicals, Poole, UK). The author is grateful for the assistance of Dr R Sharpe and Mr M Millar in preparing this tissue.

The tissue used for the experiments within this work was identical to that used by Kelnar et al (2002), and was therefore archival in nature. The reason for this relates to the availability of marmoset tissue, and highlights one of the difficulties in conducting such work with primates.

3.3 Immunohistochemistry

Details of all antibodies used in this study, including dilutions and methods used for immunohistochemistry, are shown in Table 3.1. The primary antibodies used had not been previously applied to marmoset testis. Therefore, in order to establish the most appropriate method, each antibody was initially evaluated using a range of dilutions and under differing conditions of antigen retrieval and detection. All incubations were performed at room temperature, unless otherwise stated.

Table 3.1 Primary Antibodies used

Primary antibody	Source	Species raised in	Antigen retrieval	Dilution	Secondary antibody	Avidin-Biotin block
MAGE-57B	Gift	Mouse monoclonal	No	1 in 20	Mouse Envision	No
Ki67	DAKO –M7240	Mouse monoclonal	Citrate	1 in 40	Mouse Envision	No
Phospho-histone H3	Upstate Biotech #06-570	Rabbit polyclonal	Citrate	1 in 3000	Rabbit Envision	No
EGF receptor	Sigma E3138	Mouse monoclonal	No	1 in 100	Mouse Envision	No
GFR- α 2	Santa Cruz sc-7136	Goat polyclonal	No	1 in 30	Rabbit anti-goat biotinylated	Yes
GDNF	Santa Cruz sc-328	Rabbit polyclonal	No	1 in 100	Rabbit Envision	No
Neurturin	Santa Cruz sc-8173	Goat polyclonal	No	1 in 20	Rabbit anti-goat biotinylated	Yes
IGF-1 receptor α	Santa Cruz sc-712	Rabbit polyclonal	No	1 in 200	Rabbit Envision	No
LIF receptor	Santa Cruz sc-659	Rabbit polyclonal	No	1 in 100	Swine anti-rabbit biotinylated	Yes
FGF Receptor 1	Santa Cruz sc-121	Rabbit polyclonal	No	1 in 40	Rabbit Envision	No
FGF-4	Santa Cruz sc-1361	Goat polyclonal	No	1 in 20	Rabbit anti-goat biotinylated	Yes
GM-CSF receptor	Santa Cruz sc-458	Mouse monoclonal	Citrate/ Glycine	1 in 20	Mouse Envision	No
Activin receptor IIA	R Anderson		No	1 in 600	Rabbit Envision	No
PCNA	DAKO –M0879	Rabbit polyclonal	No	1 in 100	Rabbit anti-mouse biotinylated	Yes
C-Kit	DAKO -A4502	Rabbit polyclonal	No	1 in 50	Swine anti-rabbit biotinylated	Yes
Oct-4	Santa Cruz sc-9081	Rabbit polyclonal	Citrate	1 in 200	Swine anti-rabbit biotinylated	Yes
GFR- α 1	Santa Cruz sc-6156	Goat polyclonal	No	1 in 20	Rabbit anti-goat biotinylated	Yes

Sections were dewaxed in xylene (BDH, Dorset, UK), rehydrated in graded ethanols and washed in water. As detailed in Table 3.1, sections were, if required, subjected to a heat-induced antigen retrieval step (Norton *et al*, 1994). As discussed previously, the requirement for antigen retrieval was, at the outset, uncertain for the majority of antibodies tested. Therefore each primary antibody was initially investigated both with and without antigen retrieval. Buffers used for the antigen retrieval process were 0.01M citrate (pH 6.0) and 0.05M glycine (pH 3.5). After pressure-cooking in buffer for 5 minutes at full pressure, sections were left to stand for 20 minutes before cooling under running tap water. The optimal preparation of the slides was therefore established for each primary antibody investigated. In general, staining for antigens expressed within the nucleus required this procedure, whereas the expression of cytoplasmic and peri-membranous antigens, such as growth factor receptors, did not.

Endogenous peroxidase activity was blocked by immersing all sections in 3% (v/v) H₂O₂ in methanol (both from BDH Laboratory Supplies, Poole, Dorset, UK) for 30 minutes. After washing in water, the slides were transferred into Tris-buffered saline (TBS: 0.05M Tris-HCl, 0.85% NaCl, pH 7.4) for two 5-minute washes.

To block non-specific binding sites, sections were incubated for 30 minutes with the appropriate normal serum diluted 1:5 in TBS containing 5% (w/v) bovine serum albumin (BSA: Sigma, Poole, Dorset, UK). Normal rabbit serum (NRS) was used for MAGE, Ki67, EGF receptor, the GDNF family of receptors, neurturin, FGF-4 and PCNA; normal goat serum for FGF receptor and GM-CSF receptor; and normal swine serum for phosphohistone H3, IGF-1 receptor, Activin receptor, GDNF, LIF receptor, Oct-4 and C-Kit.

Immunostaining with certain antibodies, as detailed in Table 3.1, also required blocking of endogenous biotin using an avidin/biotin blocking kit (Vector Laboratories Inc., Peterborough, UK). The primary antibodies that required an avidin/biotin block were either raised in goat, for which an Envision detection system is not available, or were shown to have improved detection using a biotinylated secondary antibody as opposed to the appropriate Envision method. The addition of avidin was incorporated into the BSA block at a dilution of 8 drops per ml, after which the sections were transferred into TBS for two 5-minute washes. Sections were then incubated in biotin, at a dilution of 8 drops per ml in TBS, before two further 5-minute washes in TBS.

Primary antibodies were then added to the sections at the optimal dilution (Table 3.1), in the appropriate normal serum in 5% TBS/BSA. Because the optimal dilution was initially not known for each primary antibody, a range of concentrations was applied to the tissue. The most appropriate antibody concentration was then selected, based on the intensity of staining and the level of background observed throughout this range. Following the application of primary antibody, sections were incubated overnight at 4°C in a humidified chamber.

Sections were then washed in TBS. Due to problems encountered with heavy background staining, sections for immunolocalization of growth factor receptors were initially washed in TBS containing 0.05% polyoxyethylene sorbitan monolaurate ('Tween' - Sigma, Poole, Dorset, UK) before the second TBS wash. All sections were then incubated for 30 minutes in the appropriate secondary antibody (Table 3.1),

diluted in the same normal serum as the primary antibody. Sections were then washed twice in TBS, which included an initial wash in TBS with 0.05% Tween for those sections stained for growth factor receptors. Those sections that had required an avidin/biotin block were then incubated for 30 minutes with avidin-biotin conjugated to horseradish peroxidase (HRP) (Vector Laboratories, as above), diluted in 0.05M Tris-HCl, pH 7.4, according to the manufacturer's instructions. All sections were then washed twice (5 minutes each) in TBS.

Bound antibody was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB) (DAKO) until staining was optimal, at which time the reaction was stopped by immersing sections in distilled water. All sections were then counterstained with haematoxylin, dehydrated in graded ethanols, cleared in xylene and coverslipped using Pertex mounting medium (CellPath plc, Hemel Hempstead, UK).

Negative controls, omitting the primary antisera, were included in each experiment. Due to limited availability of tissue, these sections were frequently from adult testis rather than younger ages. However, if this was the case adult sections were also included within the experiment in order to ensure lack of immunoexpression was due to omission of primary antibody rather than reflecting the pattern of expression at this age.

3.4 Immunofluorescence

Immunofluorescence was used for double staining the growth factor receptors with markers of proliferation. Based on the results from the initial immunohistochemical studies, the growth factor receptors selected for further investigation were the EGF

receptor and a member of the GDNF family of receptors, GFR- α 2. Co-localisation of these receptors was attempted with Proliferating Cell Nuclear Antigen (PCNA) as a marker of proliferation. This was used in addition to both Ki67 and phosphohistone H3, as these latter antigens require a heat-induced retrieval step, which affected the quality of staining of the growth factor receptors. Detection of PCNA does not require this process, and has been used as a proliferative marker in previous studies on the marmoset (Kelnar *et al*, 2002).

Sections were prepared as described previously, with incubation for 30 minutes in normal rabbit serum prior to the application of PCNA antibody (Santa Cruz, sc-7907, rabbit polyclonal) at a dilution of 1:20 in normal swine serum. Following incubation overnight at 4°C, the sections were washed in TBS as above and PCNA was visualised by Streptavidin alexa-488 (Molecular Probes Inc., Eugene, OR97402, USA) via a swine anti-rabbit biotinylated secondary antibody. Antibody to either the EGF receptor or GFR- α 2 was then applied, both at a dilution of 1:75. This optimal dilution was obtained after testing a range of antibody concentrations. Sections were incubated overnight at 4°C and following washes in TBS (with 0.05% Tween as above), the receptors were visualised by a tyramide enhanced Cy5 via mouse Envision (DAKO) for the EGF receptor and rabbit anti-goat peroxidase (Sigma, A5420) for the GFR- α 2. All sections were then counterstained with propidium iodide (Sigma, 1:2000 in Phosphate Buffered Saline), and mounted in Permafluor.

For co-localisation with Ki67 or phosphohistone H3, sections were initially subjected to a heat-induced antigen retrieval step in 0.01M citrate (pH 6.0), as discussed.

Primary antibody was applied following incubation in normal swine serum for 30

minutes. Because both the EGF receptor antibody and the Ki67 antibody were raised in mouse, an alternative Ki67 antibody was required. A polyclonal antibody raised in rabbit (DAKO, A0047) was therefore used, at a dilution of 1:50. The antibody to Phosphohistone H3 was as before, used at a dilution of 1:300. Following incubation overnight at 4°C, the sections were then treated as above, with the application of antibody to either the EGF receptor or GFR- α 2.

3.5 Double staining using Fast Blue

Double staining using putative stem cell markers was attempted with confocal microscopy, but analysis was problematic because of the small number of positive cells. Therefore double staining using light microscopy was also used. Following visualisation of the stem cell marker with DAB, sections were prepared for labelling with antibody to Ki67 as described in section 3.3, although the latter antibody was used at a dilution of 1:5 overnight to improve detection. Following two 5-minute washes in TBS, sections were incubated for 30 minutes in anti-mouse alkaline phosphatase Envision (DAKO). Sections were subsequently washed in TBS (2 x 5 minutes) and bound antibody was visualized with Fast Blue (Sigma), before mounting in Permafluor.

3.6 Image Analysis

Non-fluorescent images were photographed using a Provis microscope (Olympus Optical, London, UK) fitted with a Kodak DCS330 digital camera (Eastman Kodak, Rochester, NY, USA). Fluorescent images were captured using a Zeiss LSM Axiovert 100M confocal microscope (Carl Zeiss Ltd, Welwyn Garden City, UK).

Images were compiled using Photoshop 7.0 (Adobe Systems Inc., Mountain View, CA, USA) before being printed using a Hewlett-Packard laser jet 4600dn printer.

The immunoexpression of growth factor receptors was initially evaluated in adult testis, in order to assess the full range of germ cells that expressed particular receptors during normal spermatogenesis. This information, together with the characteristics of immunopositive cells at 35 weeks of age, enabled better interpretation of receptor expression prepubertally. It is emphasised that certain conclusions regarding immunoexpression within marmoset testis are based on qualitative information, rather than cell quantification as detailed below. The photomicrographs presented are merely examples in order to illustrate these conclusions. However, where appropriate cell quantification is used to substantiate this analysis.

3.7 Cell Quantification

Cell counts of MAGE positive cells and those stained for the proliferative markers, Ki67 and Phosphohistone H3, were performed at 35 weeks of age, both for control animals and those treated previously with a GnRH antagonist. Due to wide inter-animal variability, paired data obtained from control and GnRH antagonist-treated co-twins ($n=4$) are presented. However, due to the small number of cells labelled with Phosphohistone H3, analysis of 35 week old singleton animals (controls, $n=3$; treated, $n=5$) was also included, in addition to paired analysis of co-twins.

For analysis of the effect of suppression of the neonatal testosterone surge, cell counts were also performed for MAGE at 4 weeks of age, both for control animals and those

treated previously with a GnRH antagonist. As above, paired data from control and GnRH antagonist-treated co-twins were obtained ($n=4$).

Using a systematic clock-face sampling pattern, selected fields were viewed at 20x magnification under a light microscope (Zeiss Axiostar plus). Tubules were selected, also on the basis of a clock-face pattern, and total numbers of stained and unstained cells per tubule were obtained. From this, a mean ratio of stained to unstained cells per tubule was calculated for each animal. This approach was chosen in preference to calculating total cells per testis, because previous studies (Kelnar *et al*, 2002) have demonstrated that total germ cell number is reduced in GnRH antagonist-treated animals, whereas the spermatogonial labelling index is not. Therefore, to avoid being misled by changes in total germ cell number, calculating the ratio of labelled to unlabelled cells per tubule allowed a proliferation index to be determined. All immunopositive cells were noted to be germ cells, with no staining of Sertoli cells.

Quantification of germ cells expressing the GFR- $\alpha 2$ receptor was also performed for both control and GnRH antagonist-treated animals. Sections were examined under oil-immersion using a Leitz 63x objective fitted to an Olympus BH2 microscope, and images were analysed using Image Pro-plus version 4.5.1 with Stereology Pro 5.0 (Media Cybernetics). Twenty randomly selected fields were examined and from a 900-point grid, points falling over germ cell nuclei were categorised as either stained or unstained. These were expressed as a percentage of the total points counted. Measurement of the mean nuclear diameter for both stained and unstained germ cells, in combination with testicular weights, enabled cell counts to be expressed as total per testis.

3.8 Statistical analysis

With regards to MAGE and Ki67 staining, a mean ratio of immunopositive to immunonegative cells per tubule was calculated for each animal. Each control animal was compared with its treated co-twin. The means of the control co-twins ($n=4$) and of the treated co-twins ($n=4$) are also presented, with an error bar of 1 standard deviation. With regards to Phosphohistone H3 staining, a mean ratio of immunopositive to immunonegative germ cells per tubule was calculated for each animal. Each control animal was compared with its treated co-twin, and means from each group are presented in a similar manner to the above. However, because phosphohistone is expressed within a much narrower window, and therefore significantly fewer cells will be immunopositive than compared to a marker such as Ki67, data from singleton animals were included in addition. Therefore a mean ratio of stained to unstained germ cells for both the total control group ($n=7$) and the total treated group ($n=9$) is presented, together with error bars of 1 standard deviation.

Immunostaining for the GFR- $\alpha 2$ receptor is expressed as a ratio of the total stained germ cells per testis compared to total unstained germ cells per testis. Paired data from co-twins are presented ($n=4$), together with means from each group and error bars of one standard deviation.

Chapter 4

Results- Germ Cell Numbers and Proliferation

4.1 Introduction

The first part of this study aimed to investigate the hypothesis that spermatogonial proliferation in the prepubertal marmoset is gonadotrophin independent. Kelnar *et al* (2002) demonstrated that the proliferation of germ cells in this animal model, as assessed by the PCNA labelling index of spermatogonia, was unaffected by prior treatment with a GnRH antagonist. If this finding could be confirmed, using other markers of cell proliferation, this may explain why suppression of the gonadotrophin axis has not been successful in alleviating the gonadotoxic damage secondary to cancer treatment.

4.2 Methods

In order to investigate this hypothesis, the immunoexpression of markers of germ cell number and proliferation was studied in the prepubertal marmoset testis. This expression was then compared to testes from prepubertal animals that had previously received treatment with a GnRH antagonist.

4.2.1 Animals and Treatments

Animals used for study were 35-week-old marmosets, as detailed in chapter 3. Because of inter-animal variability, four sets of co-twins were investigated, with one twin having received GnRH antagonist treatment between 25 and 35 weeks of age, whilst the other twin acted as a control.

In addition to investigating the role of gonadotrophins in the regulation of spermatogonial proliferation during 'infancy', the effects, if any, of the neonatal testosterone surge on germ cell number and proliferation, based on the immunoexpression of the same markers, was also studied. Paired analysis of four-week-old co-twins ($n=4$) was employed, with one twin having received a GnRH antagonist since birth, whilst the other twin acted as a control.

As discussed in chapter 3, it was expected that fewer cells would be immunopositive for phosphohistone as compared to Ki67. In view of this, singleton animals were also included in the analysis of the immunoexpression of phosphohistone, in order to improve statistical significance.

4.2.2 Immunohistochemical markers

MAGE-A4, Ki67 and Phosphohistone H3 were used as markers to investigate germ cell number and proliferation, and to enable comparison of spermatogonial proliferation in control animals with those treated with a GnRH antagonist.

The melanoma-associated antigen (MAGE) is expressed both within the normal testis and in certain testicular neoplasms (Aubry *et al*, 2001). Positive immunostaining for MAGE-A4 has been demonstrated in gonocytes and spermatogonia, with weak staining in spermatocytes. Spermatids, Sertoli and Leydig cells are all immunonegative (Yakirevich *et al*, 2003). It therefore represents a useful marker for the detection and quantification of spermatogonial, and earlier, germ cell types.

Ki67 is a nuclear antigen expressed in all phases of the cell cycle, except G₀ and early G₁ (Gerdes *et al*, 1984), and has been widely used as an immunohistochemical marker of cellular proliferation. Phosphorylated Phosphohistone H3 is expressed in cells from prophase to telophase (Juan *et al*, 1998), and thus represents a marker of mitosis. This latter marker is more specific than Ki67 as it will not detect cells undergoing DNA repair, but as considerably fewer cells will be immunopositive for Phosphohistone H3 than for Ki67, both antibodies were used in this study in order to increase statistical power.

4.2.3 Immunohistochemistry

The detection of these antigens within marmoset testis was performed as described in section 3.3. Mouse Envision (DAKO) was used for the detection of both MAGE and Ki67, whilst rabbit Envision (DAKO) was used for Phosphohistone H3. Both Ki67 and Phosphohistone H3 required a heat-induced antigen retrieval step prior to detection.

4.2.4 Cell Quantification

The quantification of immunopositive cells, as detailed in section 3.7, enabled a ratio of stained to unstained cells per seminiferous tubule cross-section to be calculated for each animal investigated. These ratios were compared between control and treated co-twins. Results are presented graphically, with original data tabulated in appendix A. Assessing the proportion of stained to unstained cells per tubule in this manner allows the determination of the proliferation index of germ cells within each animal. This method is chosen in preference to calculating germ cell number per testis, as the latter would be indicative of both germ cell proliferation *and* survival. As discussed

in section 3.7, previous studies (Kelnar *et al*, 2002) have demonstrated that germ cell survival is affected by GnRH antagonist treatment, presumably via effects on Sertoli cell function. In the context of this thesis, assessment of germ cell proliferation is the primary aim, as it is this that will render the germ cells susceptible to cytotoxic therapies.

4.3 Results

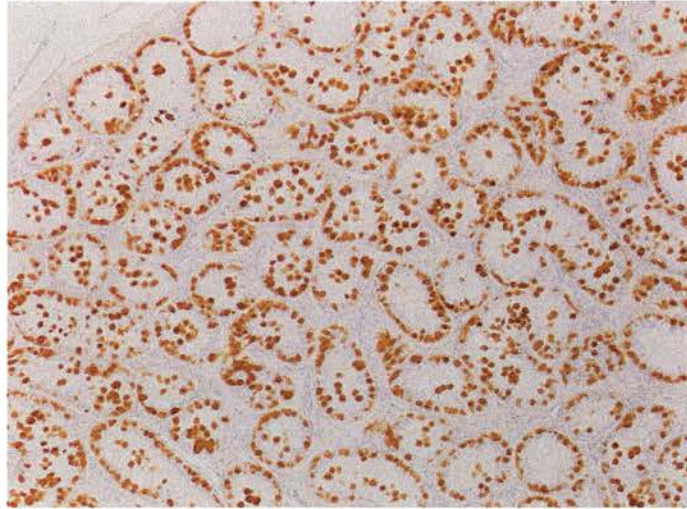
The immunoexpression of MAGE, a germ cell marker, in 35-week-old co-twins is illustrated in Figure 4.1. Numerous cells with stained nuclei were observed in the testes of prepubertal control animals, and this was unaffected by previous treatment with a GnRH antagonist. Enumeration of immunopositive cells per seminiferous tubule showed no significant difference between control and treated co-twins (Figure 4.2). It is important to note that GnRH antagonist treatment has been demonstrated to cause a reduction in mean testis weight by 34% (Kelnar *et al*, 2002), and thus total germ cell numbers within the testes may have been reduced.

Figure 4.3 illustrates the immunoexpression of MAGE at 4 weeks of age, in testes from both a control animal and its co-twin, the latter having received treatment with a GnRH antagonist since shortly after birth. Suppression of the neonatal testosterone surge did not affect the testicular expression of this antigen, and enumeration of immunopositive cells per seminiferous tubule showed no significant difference between control and treated co-twins (Fig 4.4).

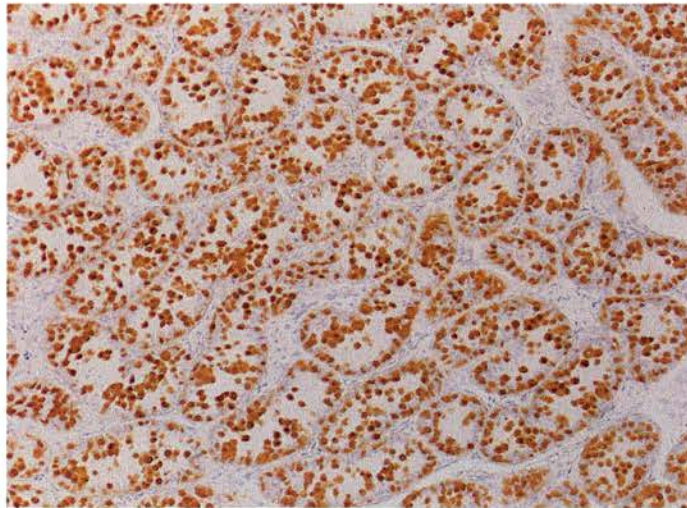
Immunoexpression of the proliferation markers, Ki67 and Phosphohistone H3, within the testes from 35-week-old animals, is demonstrated in Figures 4.5 and 4.6. As

Figure 4.1 Immunoexpression of MAGE in marmoset co-twins at 35 weeks of age
(a) control animal (b) animal treated with GnRH antagonist (c) negative control

(a) Immunoexpression of MAGE in testis from a 35 week old control animal. The numerous stained nuclei represent germ cells within the seminiferous tubules (10x magnification).



(b) Immunoexpression of MAGE in testis from the 35 week old co-twin of the above animal, which has received prior treatment with a GnRH antagonist. The numerous stained nuclei represent germ cells within the seminiferous tubules (10x magnification).



(c) Section of testis from a 35 week old animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (10x magnification). No expression is observed, in contrast to the above sections.

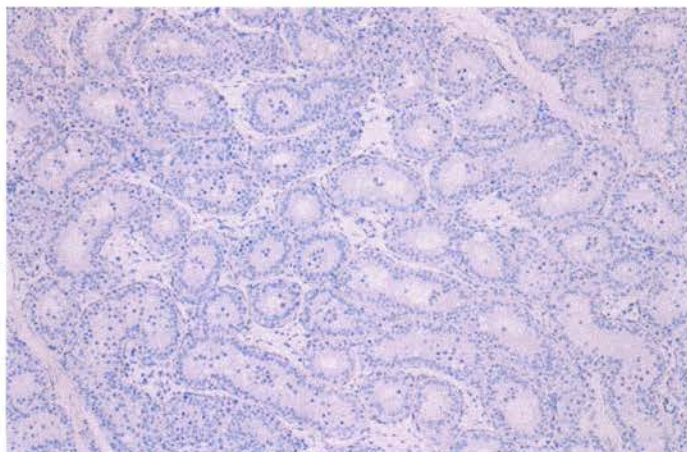


Figure 4.2 Ratio of MAGE+ :MAGE- cells per tubule in 35-week-old co-twins, treated for the previous 10 weeks with either vehicle (control) or a GnRH antagonist.

The left-hand chart illustrates the ratios for each co-twin pair, whereas the right-hand chart illustrates the means of the co-twins, with error bars of +1 standard deviation.

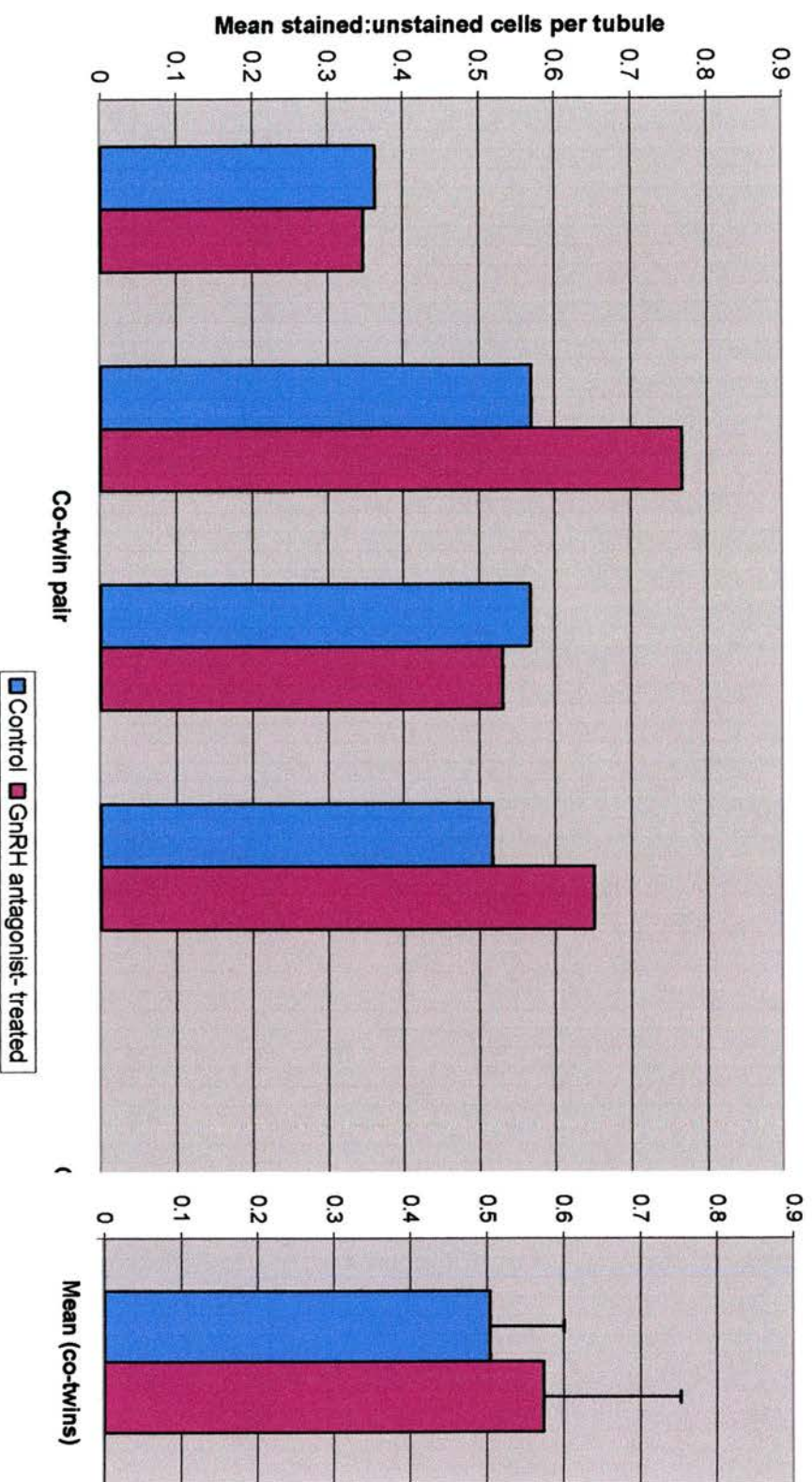
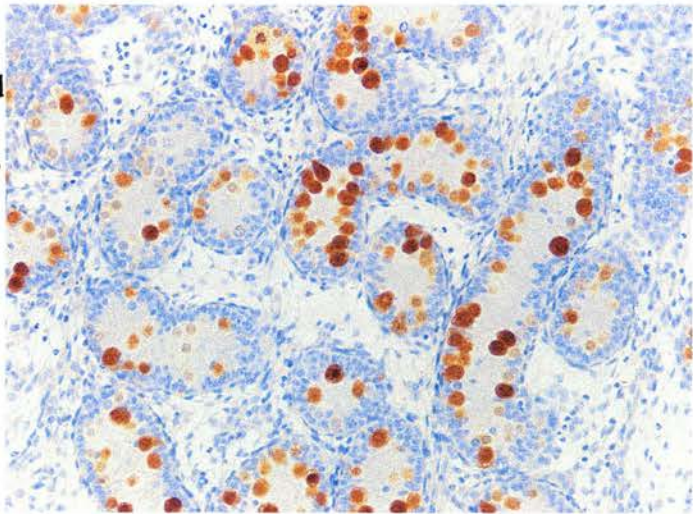
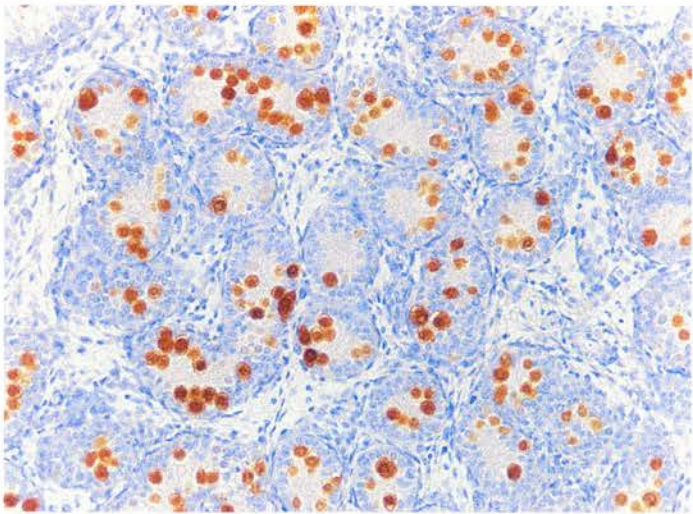


Figure 4.3 Immunoexpression of MAGE in marmoset co-twins at 4 weeks of age
(a) control animal (b) animal treated with GnRH antagonist (c) negative control

(a) Immunoexpression of MAGE in testis from a 4 week old control animal. The stained nuclei represent germ cells within the seminiferous tubules (20x magnification).



(b) Immunoexpression of MAGE in testis from the 4 week old co-twin of the above animal, which has received treatment since shortly after birth with a GnRH antagonist. The stained nuclei represent germ cells within the seminiferous tubules (20x magnification).



(c) Section of testis from a 4 week old animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (20x magnification). No expression is observed, in contrast to the above sections.

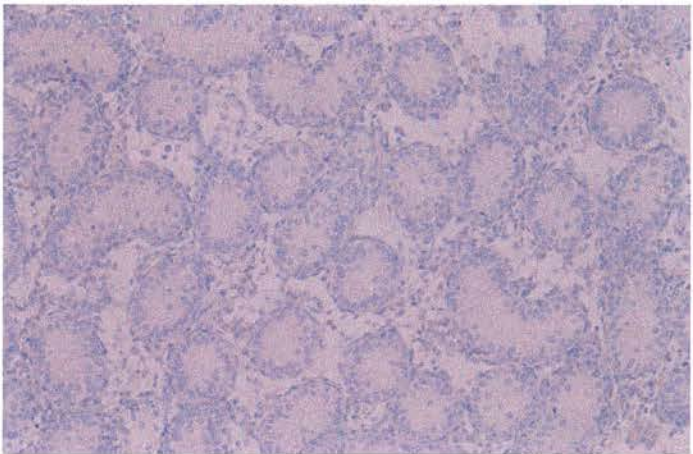


Figure 4.4 Ratio of MAGE+:MAGE- cells per tubule in 4-week-old co-twins treated since shortly after birth with either vehicle (control) or with a GnRH antagonist

The left-hand chart illustrates the ratios for each co-twin pair, whereas the right-hand chart illustrates the means of the co-twins, with error bars of +1 standard deviation.

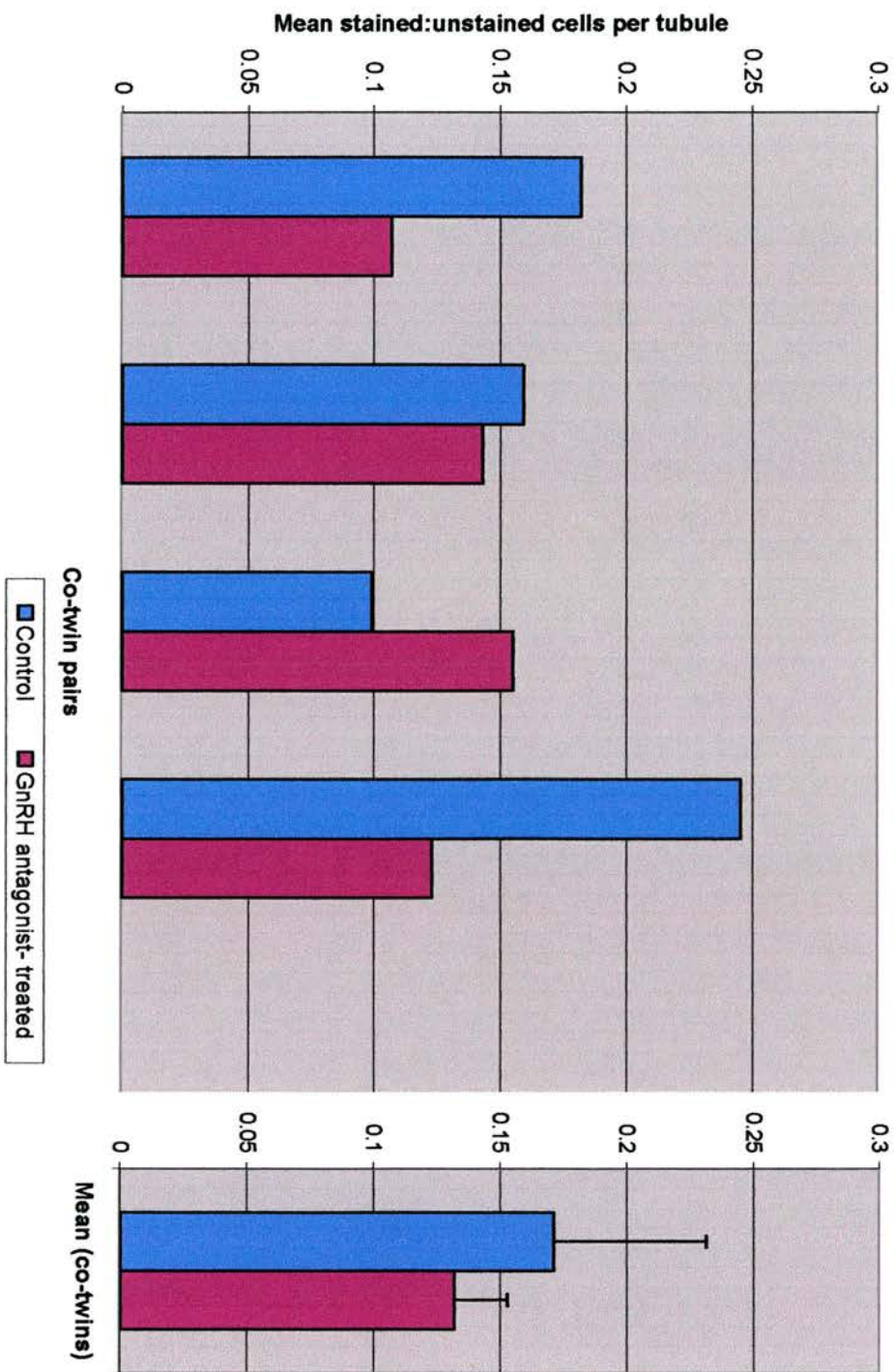
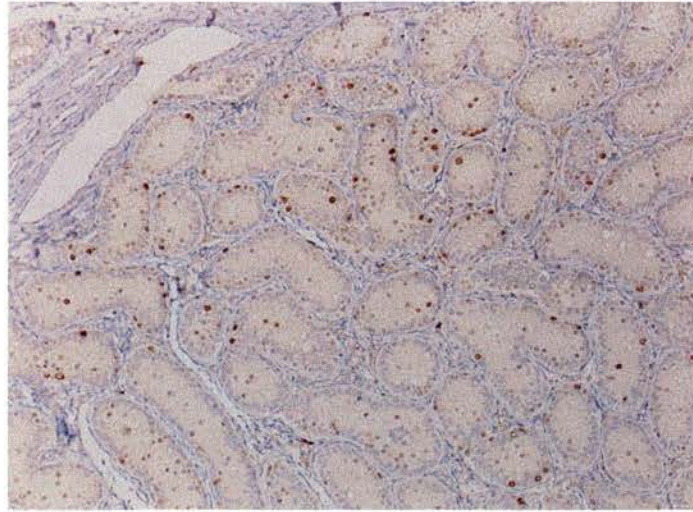
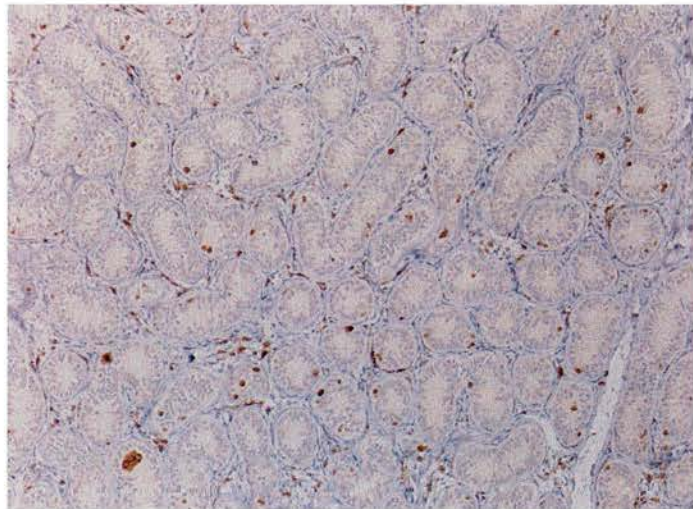


Figure 4.5 Immunoexpression of Ki67 in marmoset co-twins at 35 weeks of age
(a) control animal (b) animal treated with GnRH antagonist (c) negative control

(a) Immunoexpression of Ki67 in testis from a 35 week old control animal. The stained nuclei represent proliferating cells within the tissue, and were identified as germ cells (10x magnification).



(b) Immunoexpression of Ki67 in testis from the 35 week old co-twin of the above animal, which has received prior treatment with a GnRH antagonist. The stained nuclei represent proliferating cells within the tissue, and were identified as germ cells (10x magnification).



(c) Section of testis from a 35 week old animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (10x magnification). No expression is observed, in contrast to the above sections.

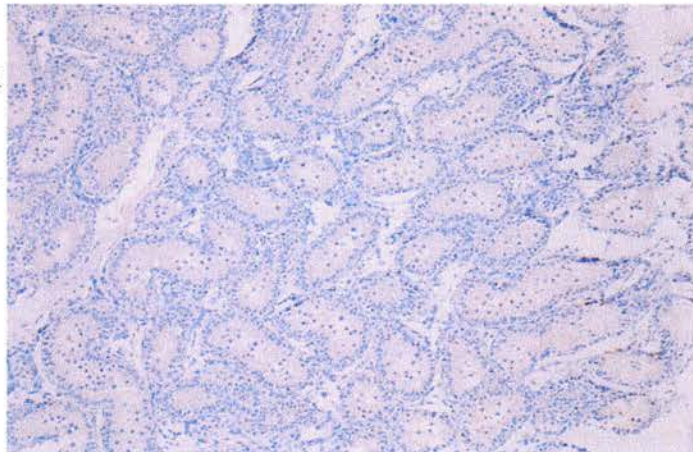
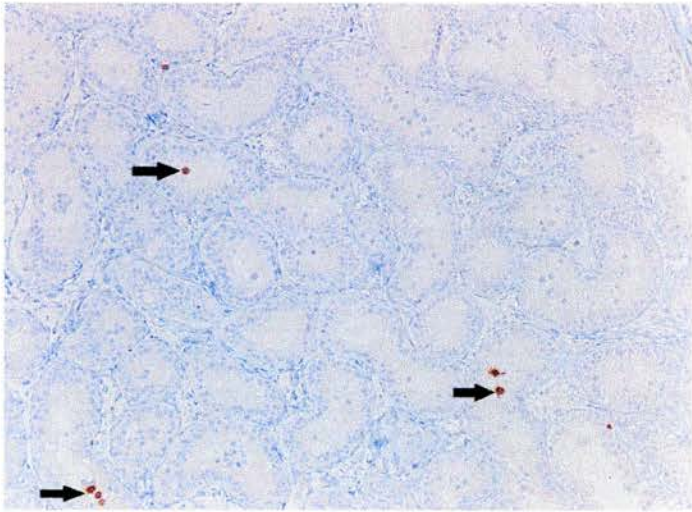
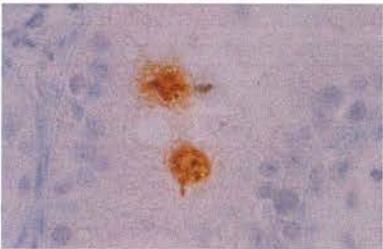
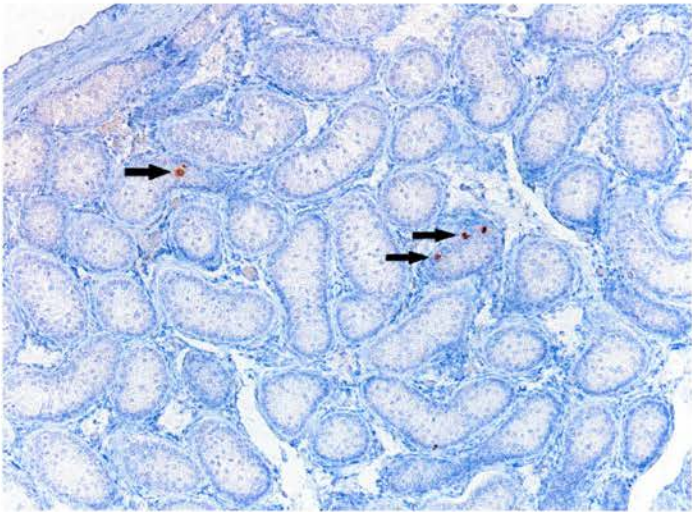


Figure 4.6 Immunoexpression of Phosphohistone H3 in marmoset co-twins at 35 weeks of age (a) control animal (b) animal treated with GnRH antagonist

(a) Immunoexpression of Phosphohistone H3 in testis from a 35 week old control animal (10x magnification). The stained nuclei (arrowed) represent proliferating cells within the tissue, and were identified as germ cells (below- 100x magnification).



(b) Immunoexpression of Phosphohistone H3 in testis from the 35 week old co-twin of the above animal, which has received prior treatment with a GnRH antagonist (10x magnification). The stained nuclei (arrowed) represent proliferating cells within the tissue, and were identified as germ cells (below- 100x magnification).



expected, fewer cells exhibited immunostaining for Phosphohistone H3 than for Ki67, due to the narrower window of expression of the former antigen. Staining for both Ki67 and Phosphohistone H3 was within the nucleus of the cell, and all positive cells were identified as germ cells, with no Sertoli cells demonstrating proliferation at this age. As with expression of the MAGE antigen, the numbers of positive cells per seminiferous tubule cross-section were not significantly different between control and treated animals (Figures 4.7 and 4.8).

Figures 4.9 and 4.10 demonstrate the immunoexpression of these proliferation markers within the testes at 4 weeks of age. Similar to the situation at 35 weeks of age, fewer cells exhibited immunostaining for Phosphohistone H3 than for Ki67. The expression of these antigens did not appear to be affected by suppression of the neonatal testosterone surge. However, because Sertoli cells are still proliferating during the neonatal period (Sharpe *et al*, 2003a), enumeration of immunopositive cells would not be a reliable indicator of germ cell proliferation, and was therefore not performed at this age.

4.4 Discussion

This part of the study aimed to investigate the hypothesis that prepubertal germ cell proliferation in the marmoset is gonadotrophin independent.

Kelnar *et al* (2002) demonstrated that the rate of proliferation of spermatogonia in the 35-week-old marmoset, based on the immunoexpression of PCNA, was not affected by prior administration of a GnRH antagonist. Here we have shown that germ cell proliferation based on the expression of further markers of proliferation, namely Ki67

Figure 4.7. Ratio of Ki67+ :Ki67- cells per tubule in 35-week-old co-twins, treated for the previous 10 weeks with either vehicle (control) or a GnRH antagonist

The left-hand chart illustrates the ratios for each co-twin pair, whereas the right-hand chart illustrates the means of the co-twins, with error bars of +1 standard deviation.

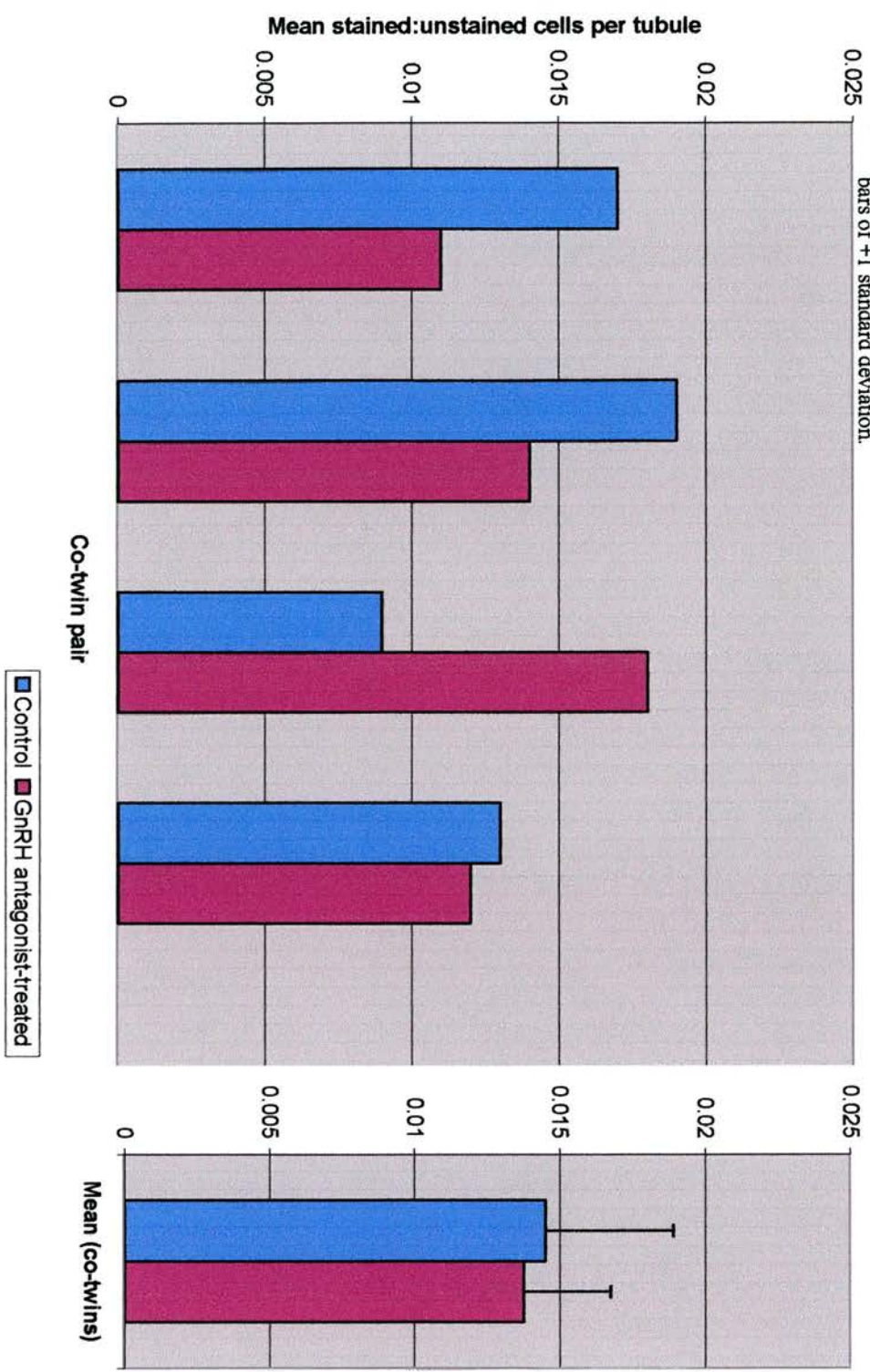


Figure 4.8 Ratio of Phosphohistone H3+ :Phosphohistone H3- cells per tubule in 35-week-old animals, treated for the previous 10 weeks with either vehicle (control) or a GnRH antagonist

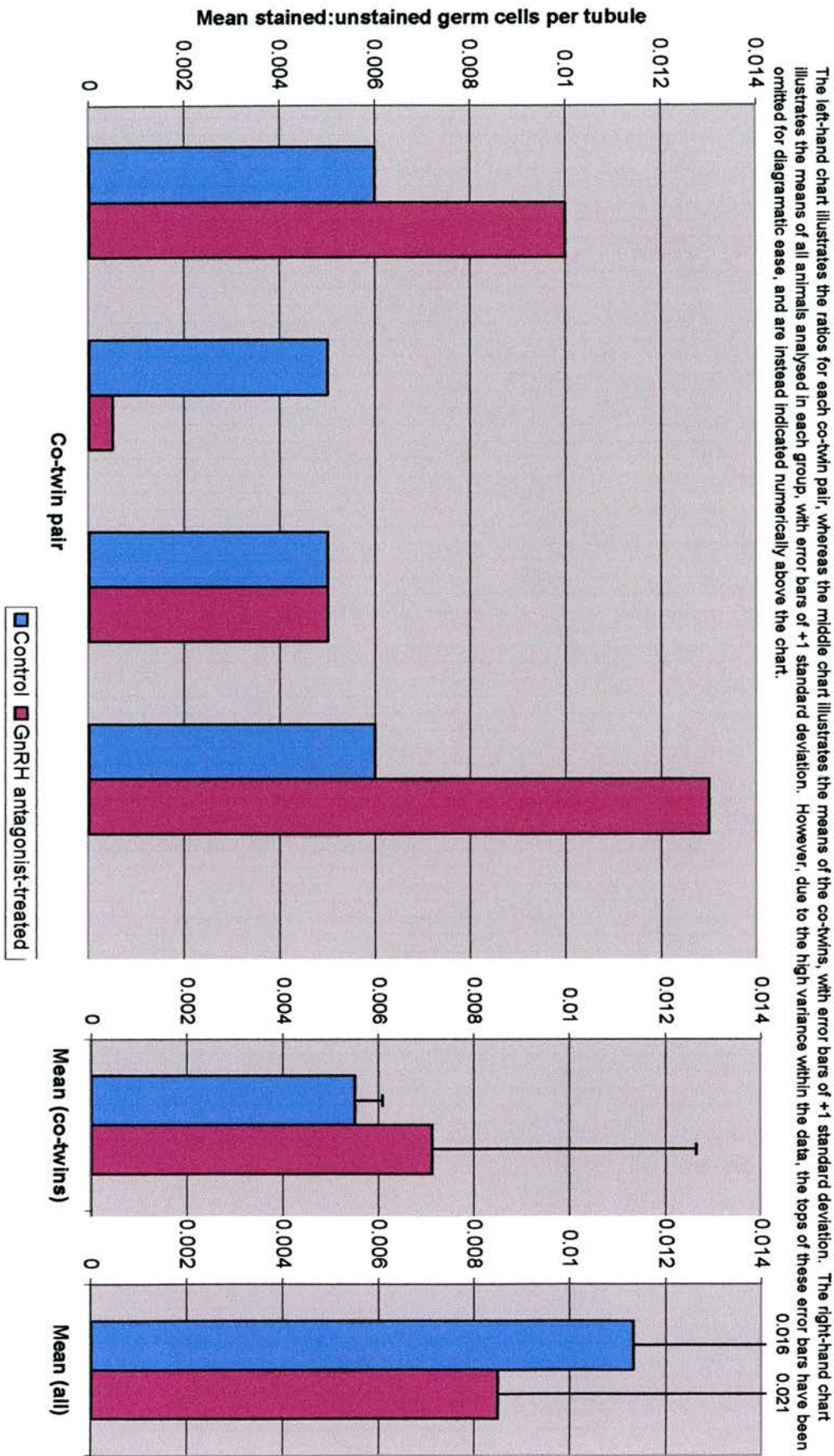
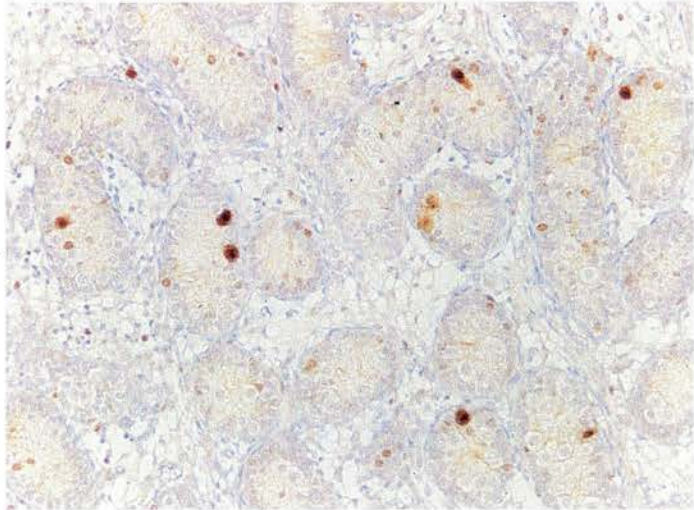


Figure 4.9 Immunoexpression of Ki67 in marmoset co-twins at 4 weeks of age
(a) control animal (b) animal treated with GnRH antagonist

(a) Immunoexpression of Ki67 in testis from a 4 week old control animal. The stained nuclei represent proliferating cells within the tissue, and will include Sertoli cells as well as germ cells (20x magnification).



(b) Immunoexpression of Ki67 in testis from the 4 week old co-twin of the above animal, which has received treatment since shortly after birth with a GnRH antagonist. The stained nuclei represent proliferating cells within the tissue, and will include Sertoli cells as well as germ cells (20x magnification).

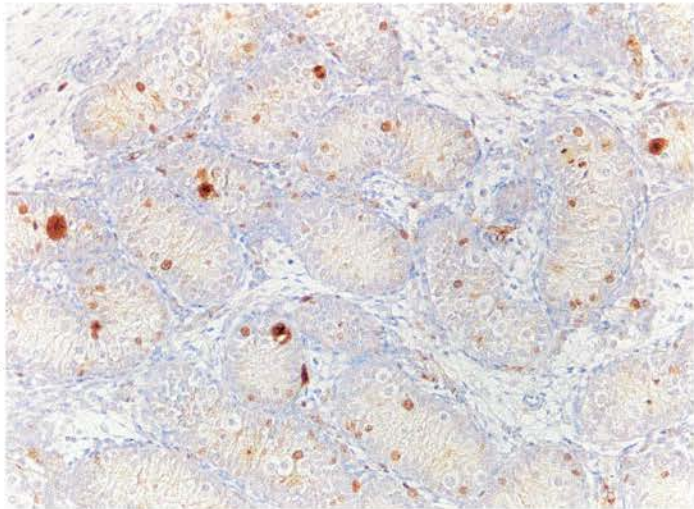
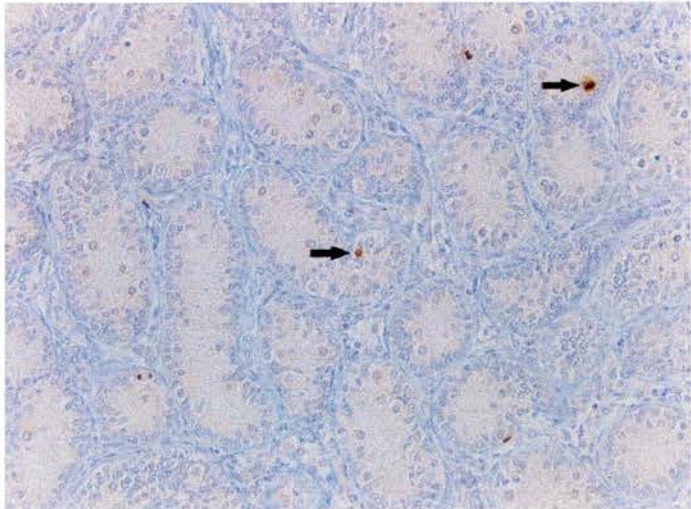
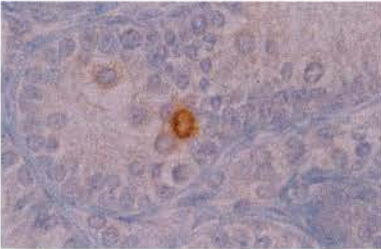
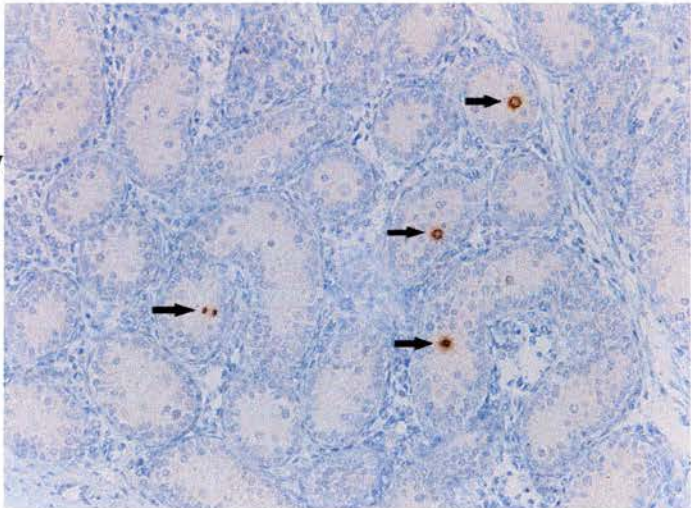
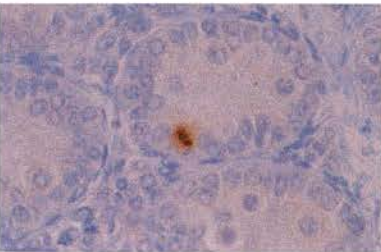


Figure 4.10 Immunoexpression of Phosphohistone H3 in marmoset co-twins at 4 weeks of age (a) control animal (b) animal treated with GnRH antagonist

(a) Immunoexpression of Phosphohistone H3 in testis from a 4 week old control animal (20x magnification). The stained nuclei (arrowed) represent proliferating cells within the tissue, and may include Sertoli cells as well as germ cells (below- 100x magnification).



(b) Immunoexpression of Phosphohistone H3 in testis from the 4 week old co-twin of the above animal, which has received treatment since shortly after birth with a GnRH antagonist (20x magnification). The stained nuclei (arrowed) represent proliferating cells within the tissue, and may include Sertoli cells as well as germ cells (below- 100x magnification).



and Phosphohistone H3, is indeed gonadotrophin independent at this age. It is reiterated that the tissue used within this study was identical to that used by Kelnar *et al* (2002), and therefore this work should ideally be repeated using alternative animals. However, the availability of suitable primate tissue restricts the opportunity for this to occur.

In addition to the above markers of proliferation we have demonstrated that, using immunoexpression of MAGE, the number of germ cells present within the seminiferous tubule, as a proportion of the total cells present, is unaffected by prior administration of GnRH antagonist. These results would suggest that spermatogonial proliferation in the prepubertal marmoset is undoubtedly gonadotrophin independent.

Despite the hypothalamic-pituitary-gonadal axis being relatively 'quiescent' during this period of testicular development, administration of a GnRH antagonist to the prepubertal marmoset between 25 and 35 weeks of age does have a detectable effect on testicular morphology and function (Kelnar *et al*, 2002). Mean testis weight is reduced by 34% and functional maturation of both Sertoli cells and Leydig cells is impaired. The total number of germ cells, including proliferating germ cells, will be reduced in the treated animals (Kelnar *et al*, 2002). However, germ cell proliferation *per se* appears unaffected by prior treatment with a GnRH antagonist. It appears that, although spermatogonial proliferation is unaffected by this treatment, fewer of these cells survive. This is presumably because they do not receive the appropriate metabolic support from the Sertoli cells, as these cells have been deprived of the hormonal stimuli required to express these functions (Kelnar *et al*, 2002).

Similarly, suppression of the neonatal testosterone surge does not appear to affect spermatogonial proliferation during this period of testicular development. Analysis of the immunoexpression of proliferation markers at 4 weeks of age is complicated by Sertoli cell replication at this age, and thus not all proliferating cells present within the seminiferous tubules are germ cells. However, this conclusion supports previous data also suggesting that spermatogonial proliferation in the neonatal period is gonadotrophin independent (Sharpe *et al*, 2003b). This latter study demonstrated that germ cell proliferation and differentiation was unaffected by suppression of the neonatal testosterone surge, although as with animals suppressed during ‘infancy’, there was impaired germ cell survival due to withdrawal of gonadotrophin support for Sertoli cells.

Suppression of the hypothalamic-pituitary-gonadal axis in adult rodents receiving chemotherapy and radiotherapy results in an improved recovery of spermatogenesis following the gonadotoxic insult (Ward *et al*, 1990; Kurdoglu *et al*, 1994). The mechanism of testicular damage following cytotoxic therapy is uncertain but appears to involve depletion of the proliferating germ cell pool, by killing cells not only at the stage of differentiating spermatogonia (Meistrich *et al*, 1982), but also stem cells themselves (Bucci and Meistrich, 1987). It is presumed that gonadotrophin suppression during cytotoxic therapy inhibits germ cell proliferation and thus reduces their susceptibility to such insults. However, as discussed previously, the precise mechanism of fertility protection in this manner remains uncertain, as it has also been demonstrated that hormonal manipulation with a GnRH antagonist results in successful recovery of spermatogenesis in rats even when given *after* the gonadotoxic insult (Meistrich and Kangasniemi, 1997).

Despite the success of these techniques in rodent models, trials of hormonal manipulation to protect testicular function in both primate models (Kamischke *et al*, 2003) and clinical studies (Thomson *et al*, 2002b) have failed to demonstrate any benefit, regardless of the timing of gonadotrophin suppression in relation to the cytotoxic insult. Although the severity of the gonadotoxic insult and the uncertainty regarding the mechanism of gonadal protection complicate interpretation of these studies, it is clear that a better understanding of spermatogenesis in the primate is needed before further clinical trials in this area. As discussed previously, significant differences exist between the organisation of spermatogenesis in rodents as compared to a number of primate species, including man, which complicates the extrapolation of data from rodent models. Our results using the marmoset as a primate model strongly suggest that prepubertal spermatogonial proliferation in this species is gonadotrophin independent, and, should the same hold true for the human, this probably explains the failure of gonadal protection based on the hypothalamic-pituitary-gonadal axis.

The identification of factors that do regulate spermatogonial proliferation would not only improve our understanding of the physiology of the developing primate testis, but may also aid in devising strategies to protect fertility in boys treated for cancer.

Chapter 5

Results- Growth Factor Receptors and Associated Ligands

5.1 Introduction

Having established that spermatogonial proliferation in the prepubertal marmoset testis is gonadotrophin independent, the next phase of the study was directed towards elucidating what factors might regulate their proliferation at this age. If these factors could be identified, firstly in this animal model and subsequently in the human testis, they would represent novel targets for gonadal protection in prepubertal boys receiving gonadotoxic cancer therapy.

As mentioned previously, a number of growth factors have been identified within rodent testes. A number of these factors have been implicated as having a role in the regulation of spermatogonial proliferation, predominantly on the basis of gene knockout models and studies of spermatogonia cultured *in vitro*. Despite the recognised differences between spermatogenesis in rodents and marmosets, these growth factors were used as a starting point for investigation.

5.2 Methods

To identify which growth factors may be involved in the regulation of germ cell proliferation in the prepubertal marmoset, their immunoexpression within the testis was investigated. In particular, the immunoexpression of their associated receptors in spermatogonia was determined.

Following the identification of potentially relevant growth factor receptors, the intention was then to co-localise these receptors with markers of cell proliferation, in order to ascertain their relationship with actively proliferating spermatogonia.

5.2.1 Animals and Treatments

Sections of testis taken from marmosets killed at 35 weeks of age were studied. To put findings from these animals into context, testes from animals killed at various time points, from the neonatal period through to adulthood, were also investigated. In addition, the immunoexpression of these growth factors and their receptors was studied in animals that had previously received treatment with a GnRH antagonist, as detailed in chapter 3. This included 35-week-old animals that had been treated with a GnRH antagonist between 25 and 35 weeks of age, and animals at 4 weeks of age in which the neonatal testosterone surge had been suppressed by such treatment. Analysis of testes from these animals would establish the gonadotrophin dependence of growth factors identified.

5.2.2 Immunohistochemical Markers

Immunohistochemical markers studied were selected on the basis of rodent studies (Tajima *et al*, 1995; Dirami *et al*, 1999; Wahab-Wahlgren *et al*, 2003; Kanatsu-Shinohara *et al*, 2003). Those studied were Insulin-like Growth Factor-1 (IGF-1), Epidermal Growth Factor (EGF), members of the Glial Cell-line Derived Neurotrophic Factor (GDNF) family, Leukaemia Inhibitory Factor (LIF), Fibroblast Growth Factor (FGF) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF).

In order to clarify certain aspects of immunoexpression of these receptors, studies were also included to investigate the presence of the associated ligands within the marmoset testis. Those that required further investigation were the GDNF family of growth factors, because cross-reactivity is reported to occur between family members (Wang *et al*, 2000), and the FGF family, in which many members signal via the same receptor (Bellosta *et al*, 2001). Thus, with regards to the GDNF family, the immunoexpression of both GDNF itself and Neurturin was studied. FGF-4 is the member of the FGF family that has been previously reported to be involved in spermatogenesis (Yamamoto *et al*, 2002), and therefore the immunoexpression of this ligand was also studied.

Activin is a member of the transforming growth factor- β family, which also includes inhibin, and is involved in the regulation of FSH secretion (de Kretser *et al*, 2001). However, *in vitro* cultures of rat germ cells with Sertoli cells have suggested that activin may also act to stimulate spermatogonial proliferation (Mather *et al*, 1990). The immunoexpression of the activin receptor in prepubertal marmoset testis was therefore also included in this study.

5.2.3 Immunohistochemistry

The immunohistochemical methods used for detecting the presence of these growth factor receptors and their associated ligands are detailed in chapter 3. Trials under differing conditions established the optimal method of detection for each primary antibody studied. As discussed previously, background staining was particularly problematic in the study of a number of these growth factor receptors in marmoset

testis, and therefore a variety of techniques were employed to reduce this, such as washing with TBS containing Tween.

On the basis of these immunohistochemical studies, growth factors potentially involved in regulating spermatogonial proliferation were selected for further investigation. Using immunofluorescence, the expression of the growth factor receptors was co-localised with markers of cell proliferation. Ki67 and Phosphohistone H3 were initially used, as these had previously been investigated in marmoset testis. However, because the heat-induced antigen retrieval step required for the detection of both of these antigens interfered with the detection of the growth factor receptors, PCNA was predominantly used as a proliferation marker for these studies.

Immunofluorescence requires more sensitive detection methods than does conventional immunohistochemistry, and therefore the problem of background staining was exacerbated.

5.3 Results

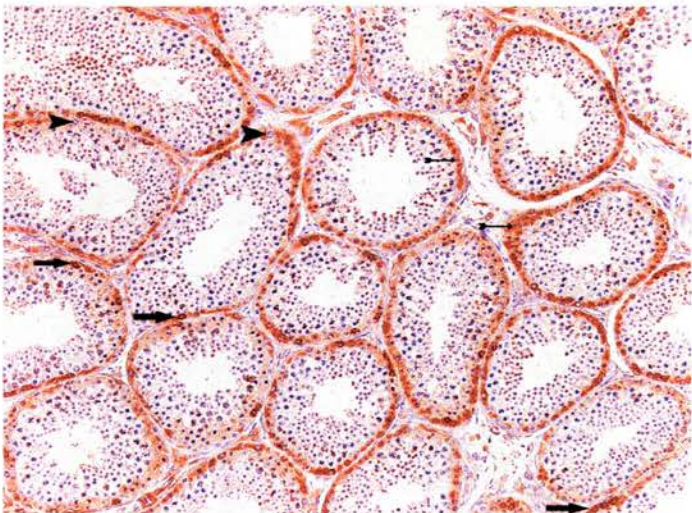
In order to aid interpretation of the expression of growth factor receptors prepubertally, immunopositive cells were initially evaluated in the adult testis, as discussed in section 3.6.

5.3.1 Immunoexpression of EGF Receptor

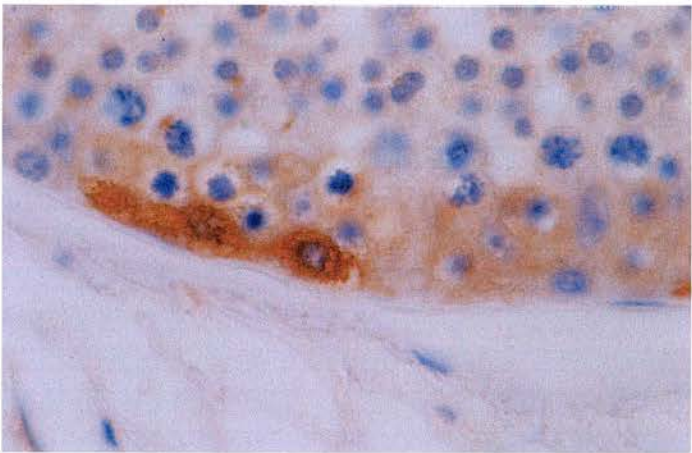
The EGF receptor was localized to the cytoplasm of a number of germ cells. Examination of its expression within the adult testis revealed intense labelling of B-

Figure 5.1 Immunoexpression of the EGF Receptor in marmoset testis
(a-c) Adult (d) 35-week-old infant (e) 35-week-old co-twin treated prepubertally
with a GnRH antagonist (f) 7-week-old neonate (g) Negative control

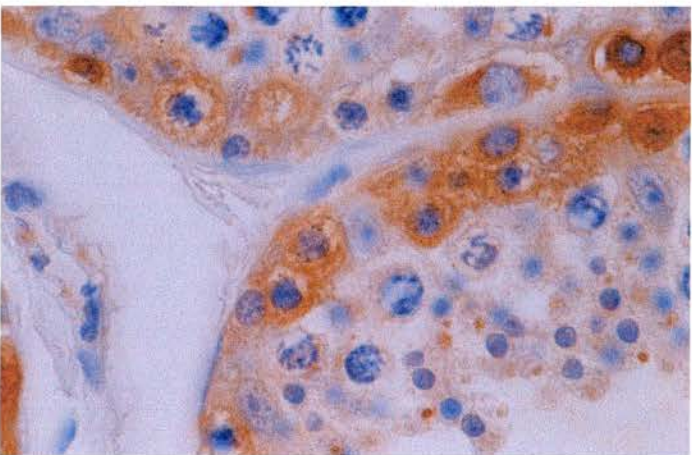
(a) Immunoexpression of the EGF receptor in adult testis. Intense staining of spermatogonia is demonstrated (solid arrows), along with less intense staining of spermatocytes (thin arrows). Occasional germ cells are immunonegative for the EGF receptor (arrowheads) (10x magnification).



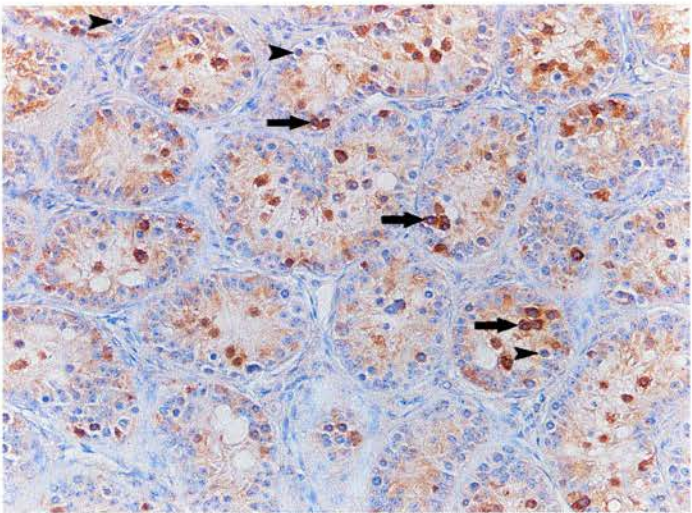
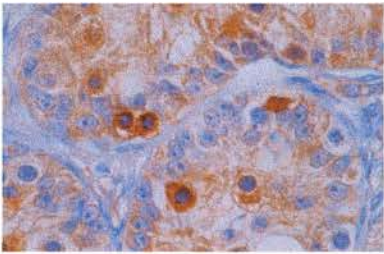
(b) Higher power photomicrograph demonstrating the intense immunoexpression of the EGF receptor in B-spermatogonia within adult marmoset testis (100x magnification).



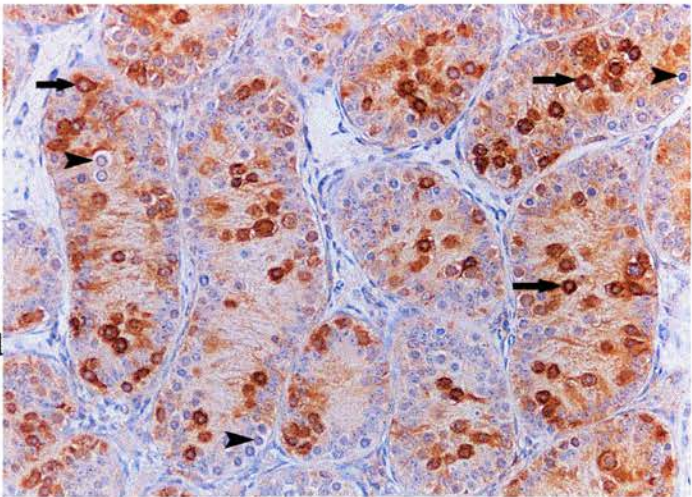
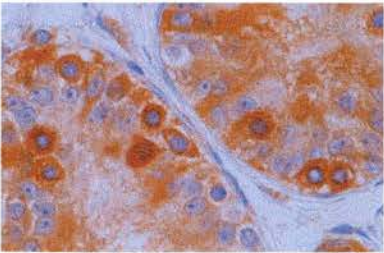
(c) Higher power photomicrograph demonstrating the less intense immunoexpression of the EGF receptor in spermatocytes within adult marmoset testis (100x magnification).



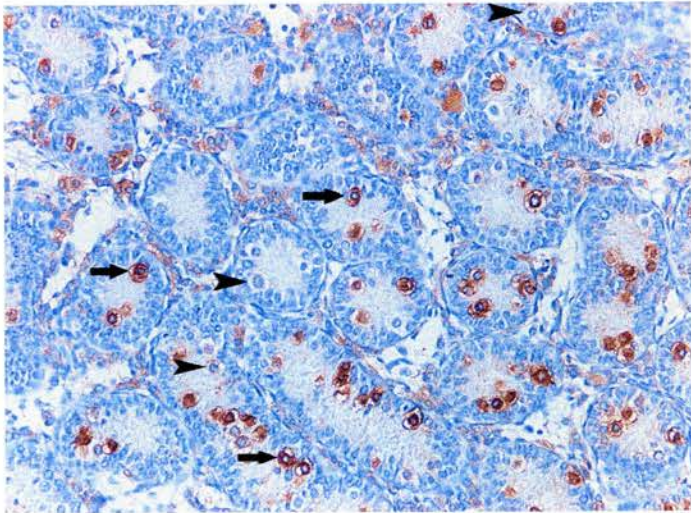
(d) Immunoexpression of the EGF receptor in testis from a 35 week old control animal (20x magnification). Numerous germ cells are immunopositive for this receptor (such as those indicated with solid arrows, and demonstrated below at 100x magnification), although a number are immunonegative (arrowheads).



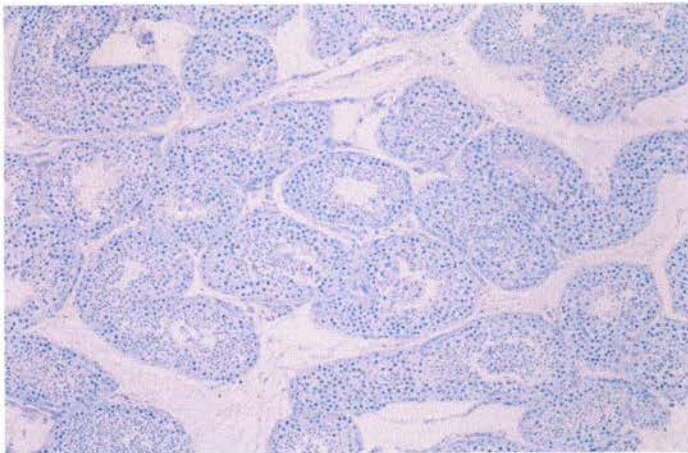
(e) Immunoexpression of the EGF receptor in testis from the 35 week old co-twin of the above animal, which has received prior treatment with a GnRH antagonist (20x magnification). A similar pattern of expression exists, with both immunopositive (solid arrows, and demonstrated below at 100x magnification) and immunonegative (arrowheads) germ cells.



(f) Immunoexpression of the EGF receptor in testis from a 7 week old control animal. A number of germ cells are immunopositive for this receptor (such as those indicated with solid arrows), although less so than at 35 weeks of age. In addition, they appear to be in a more central position within the tubule. Many germ cells are immunonegative (such as those indicated with arrowheads) (20x magnification).



(g) Section of testis from an adult animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (10x magnification). No expression is observed, in contrast to the above sections.



spermatogonia (Fig 5.1a, b). Staining of preleptotene and leptotene spermatocytes was also noted (Fig 5.1a, c), but was of reduced intensity. In addition to staining of B-spermatogonia, occasional staining of A-spermatogonia was also demonstrated.

Examination of testes from the 35-week-old animals demonstrated immunoexpression of this receptor in numerous germ cells within this age group (Fig 5.1d). However, within the same tubule, a number of germ cells were identified that were immuno-negative for this receptor. A similar pattern of expression was found in animals treated with a GnRH antagonist (Fig 5.1e). The staining for the EGF receptor observed in testes from the treated animals actually appeared to be more intense, when compared to control testes, although this finding was not consistent in all animals studied.

Based on the immunopositive cells observed in the adult, and the characteristics of the labelled cells seen, including nuclear morphology and cell size, B-spermatogonia appeared to be the predominant germ cell type that immunostained for the EGF receptor prepubertally.

Examination of testes from animals killed in the neonatal period demonstrated immunopositive germ cells, which also appear to be B-spermatogonia (Fig 5.1f). Labelled cells were less numerous than during infancy, and their position within the seminiferous tubule was noted to be more central as compared to testes from older animals.

Indeed, the position of labelled cells within the seminiferous tubules varied with age, from being central in the neonatal period to exclusively basal in adulthood. At 35 weeks of age labelled cells were noted both centrally and on the basement membrane. As discussed previously, gonocytes migrate towards the basement membrane during prenatal and neonatal life. However, at 35 weeks of age tight junctions are forming between adjacent Sertoli cells, and thus the presence of numerous germ cells in a central position is unexpected.

Quantification of immunopositive and immunonegative germ cells was not performed for the EGF receptor due to the wide window of expression, both across different ages and throughout different cell types. This is in contrast the immunoexpression of GFR- α 2, as discussed below.

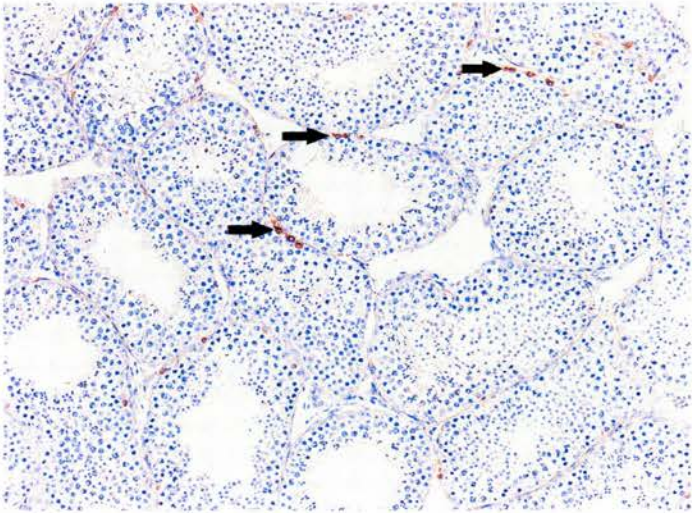
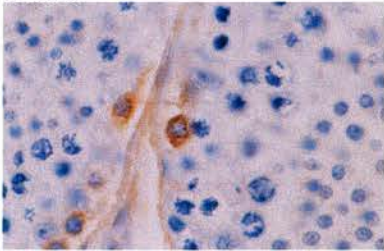
No immunoexpression is observed within the negative control slide (Fig 5.1g), which was treated identically to the other slides except for the omission of the primary antibody. As discussed in chapter 3, an adult marmoset section was used due to the availability of tissue.

5.3.2 Immunoexpression of GFR- α 2

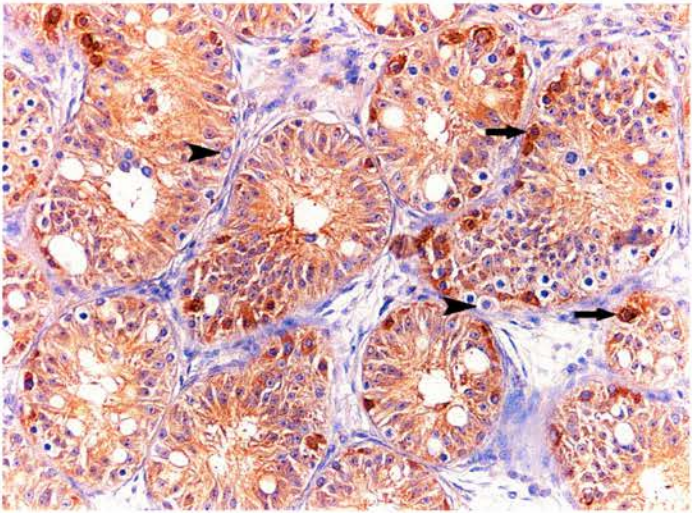
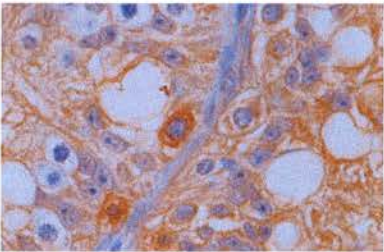
As with the EGF receptor, GFR- α 2 immunoexpression was localized to germ cell cytoplasm. Examination of its expression within the adult testis revealed intense labelling of B-spermatogonia (Fig 5.2a). However, unlike staining for the EGF receptor, staining of germ cells at more advanced stages of maturation was not found.

Figure 5.2 Immunoexpression of the GFR- α 2 receptor in marmoset testis
(a) Adult (b) 35-week-old infant (c) 35-week-old co-twin treated prepubertally
with a GnRH antagonist (d) 7-week-old neonate (e) Negative control

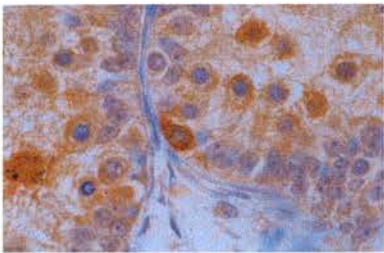
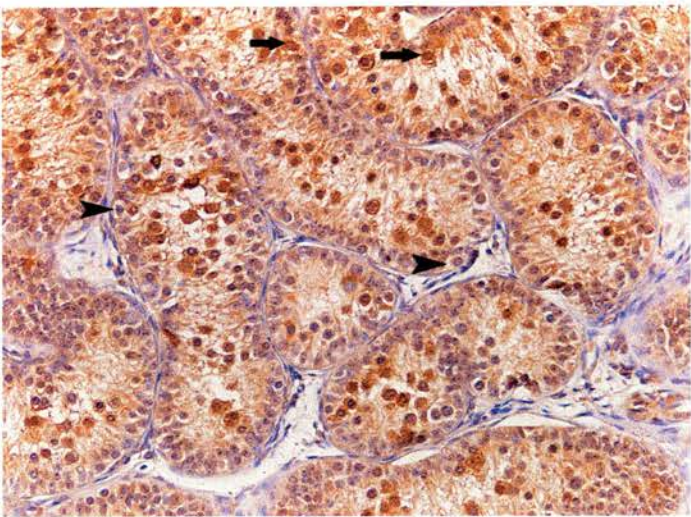
(a) Immunoexpression of GFR- α 2 in adult testis (10x magnification). Intense staining of B-spermatogonia is demonstrated (solid arrows, and below at 100x magnification). No other immunostaining is noted.



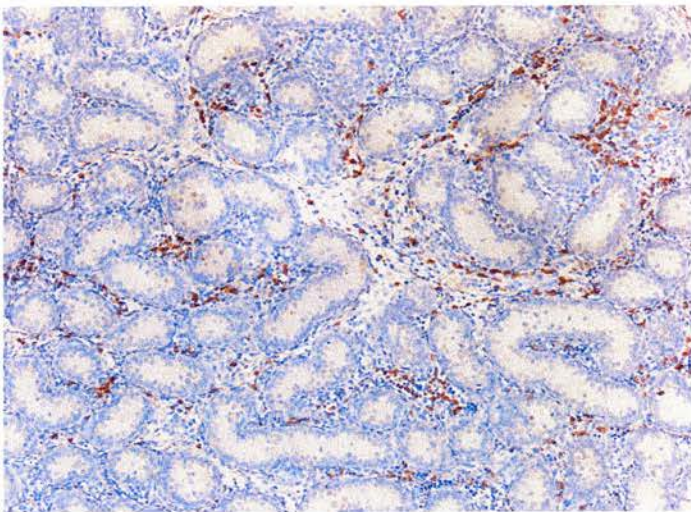
(b) Immunoexpression of GFR- α 2 in testis from a 35 week old control animal (20x magnification). Numerous germ cells are immunopositive for this receptor (such as those indicated with solid arrows, and shown below at 100x magnification), although a number are immunonegative (arrowheads).



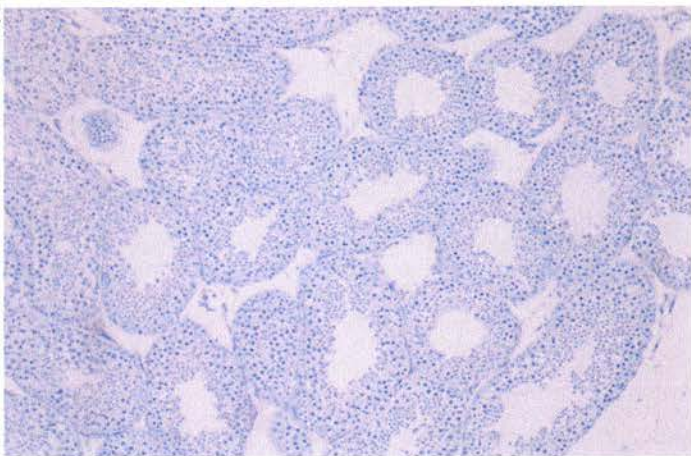
(c) Immunoexpression of GFR- $\alpha 2$ in testis from the 35 week old co-twin of the above animal (20x magnification), which has received prior treatment with a GnRH antagonist. A similar pattern of expression exists, with both immunopositive (solid arrows, shown below at 100x magnification) and immunonegative (arrowheads) germ cells.



(d) Immunoexpression of GFR- $\alpha 2$ in testis from a 7 week old control animal. No expression is seen within the seminiferous tubules, although immunoexpression is noted within the interstitium (10x magnification).



(e) Section of testis from an adult animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (10x magnification). No expression is observed, in contrast to the above sections.



Examination of testes from the 35-week-old animals demonstrated immunoexpression of GFR- α 2 in a significant proportion of germ cells within this age group (Fig 5.2b). Expression of this receptor appeared to be unaffected by previous treatment with a GnRH antagonist (Fig 5.2c). Indeed, the proportion of total germ cells stained for GFR- α 2 in control and treated testes was comparable, as demonstrated in Figure 5.3 (data tabulated in appendix B). Based on the pattern of expression in the adult, and both nuclear morphology and cell size, the germ cells expressing this receptor were predominantly B spermatogonia.

Examination of testes from animals killed in the neonatal period demonstrated no expression of GFR- α 2 within seminiferous tubules (Figure 5.2d). Indeed, the only positive immunoexpression at this age was observed in the interstitium.

No immunoexpression is observed within the negative control slide (Fig 5.2e), which was treated identically to the other slides except for the omission of the primary antibody. As discussed in chapter 3, an adult marmoset section was used due to the availability of tissue.

Because different members of the GDNF family may interact with the GFR- α 2 receptor, the immunoexpression of both GDNF and Neurturin was also investigated within the marmoset testis. Expression of these ligands was noted in a number of germ cells at 35 weeks of age (Fig 5.4). In addition, GDNF, and to a lesser extent Neurturin, was also localised to Sertoli cells. Immunoexpression of these ligands was

Figure 5.3 Ratio of GFR-a2+: GFR-a2- germ cells per testis in 35-week-old co-twins treated for the previous 10 weeks with either vehicle (control) or with a GnRH antagonist

The left-hand chart illustrates the ratios for each co-twin pair, whereas the right-hand chart illustrates the means of the co-twins, with error bars of +1 standard deviation

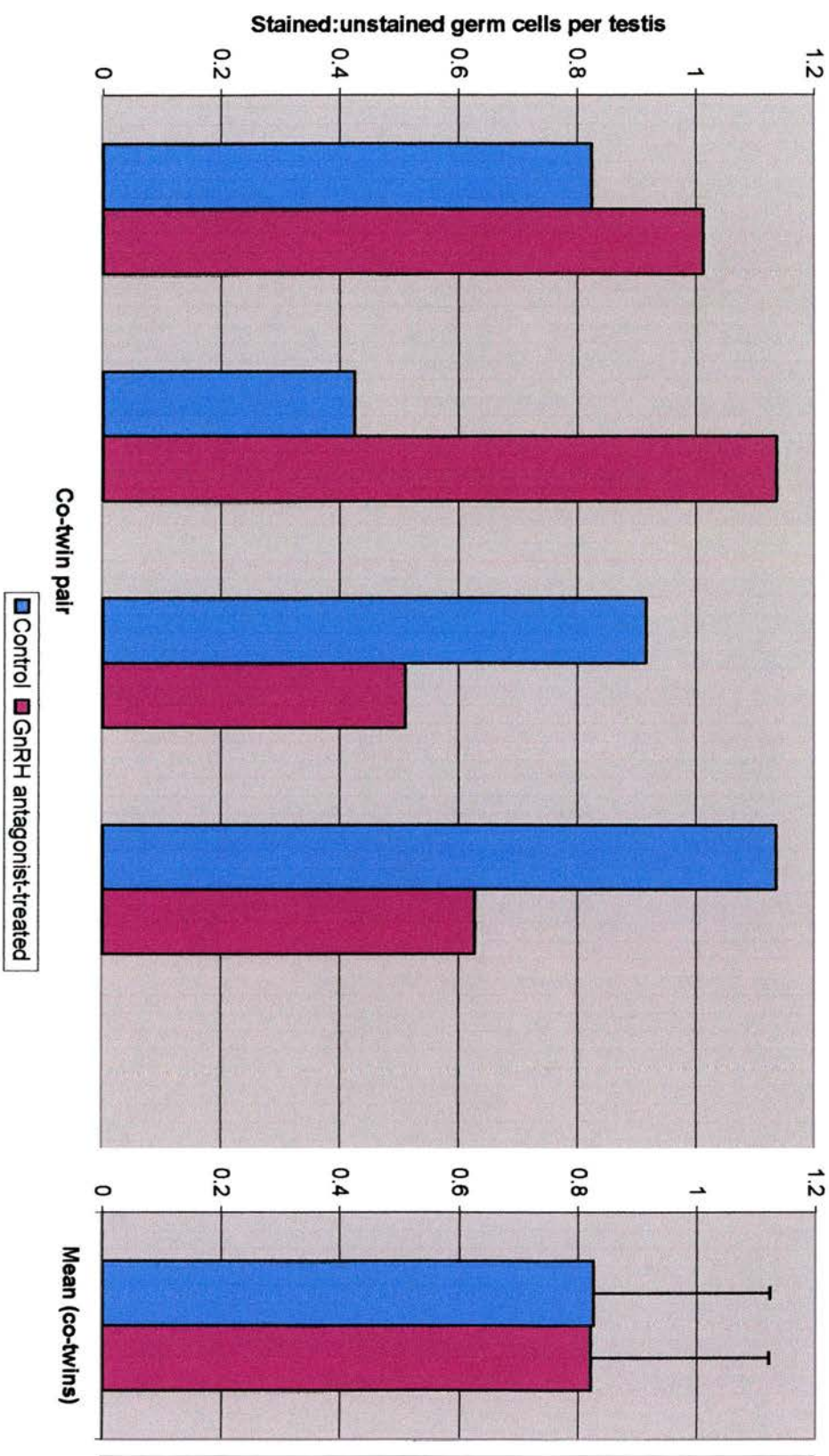
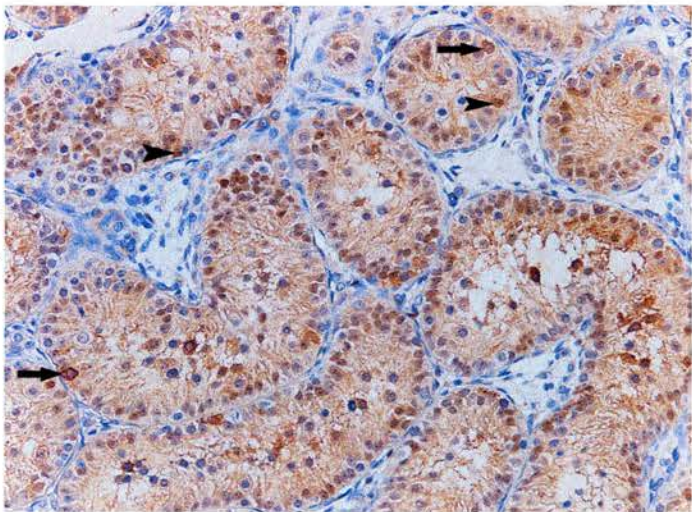


Figure 5.4 Immunoexpression of (a) GDNF and (b) Neurturin within 35-week-old marmoset testis

(a) Immunoexpression of GDNF in testis from a 35 week old control animal. A number of germ cells are immunopositive for this ligand (solid arrows), as well as Sertoli cells (indicated by arrowheads) (20x magnification).



(b) Immunoexpression of Neurturin in testis from a 35 week old control animal. A number of germ cells are immunopositive for this ligand (solid arrows), as well as occasional Sertoli cells (such as that indicated by arrowhead) (20x magnification).

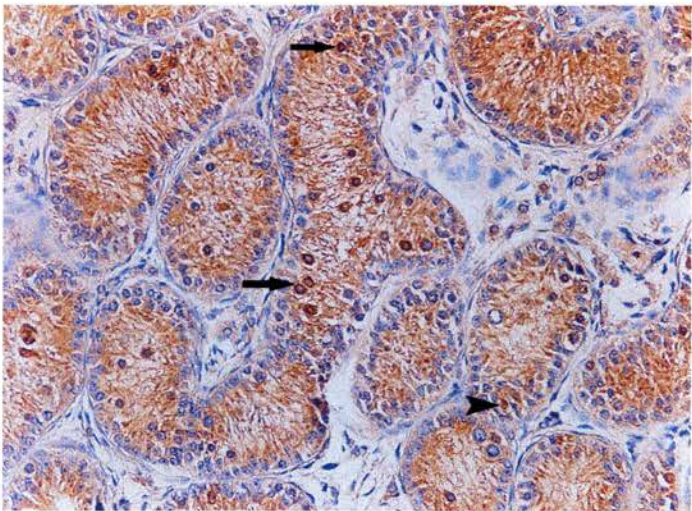
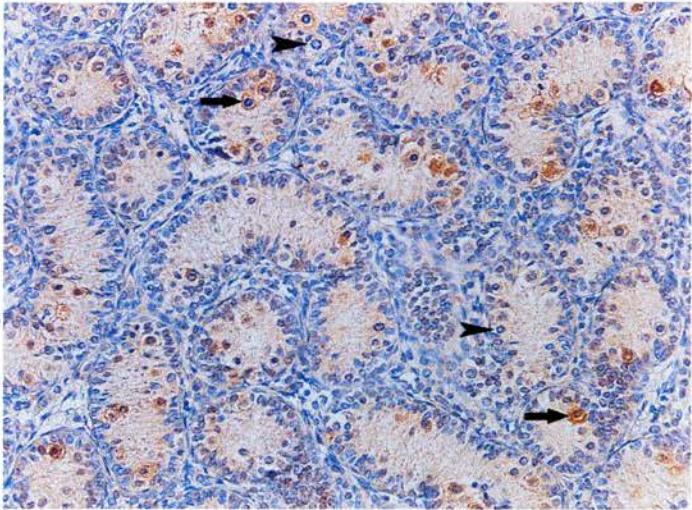


Figure 5.5 Immunoexpression of GDNF within (a) 7-week-old marmoset testis and (b) Adult marmoset testis

(a) Immunoexpression of GDNF in testis from a 7 week old control animal. Numerous germ cells are immunopositive for this ligand (solid arrows), although a number remain immunonegative (arrowheads). Unlike immunostaining for the GFR- α 2 receptor at this age, no staining is observed within the interstitium (20x magnification).



(b) Immunoexpression of GDNF in testis from an adult control animal. As with the immunoexpression of the GFR- α 2 receptor at this age, intense staining of occasional B-spermatogonia can be visualised (solid arrows). The staining observed within the lumen of occasional tubules most likely represents staining of spermatid cytoplasm, which is often encountered in postpubertal sections (10x magnification).

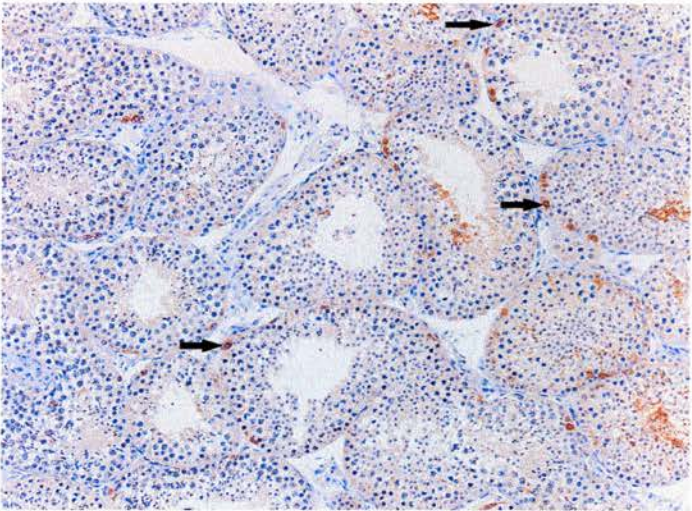
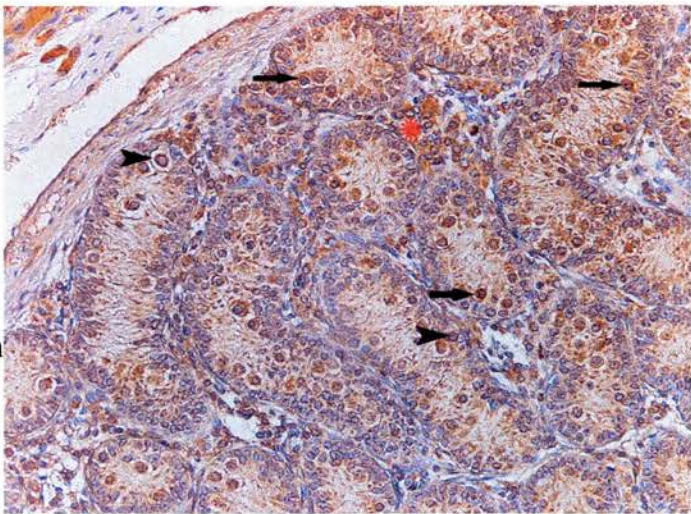
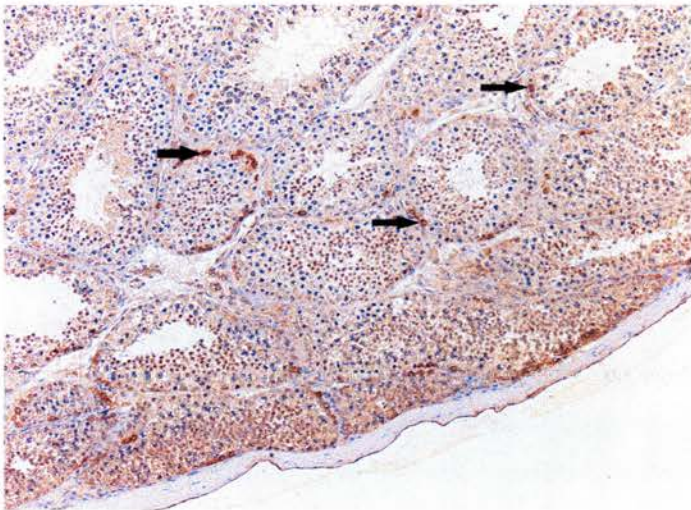


Figure 5.6 Immunoexpression of Neurturin within (a) 7-week-old marmoset testis and (b) Adult marmoset testis

(a) Immunoexpression of Neurturin in testis from a 7 week old control animal. Numerous germ cells are immunopositive for this ligand (solid arrows), although a number remain immunonegative (arrowheads). In addition, staining is observed within the interstitium (red asterisk), as noted for the GFR- α 2 receptor at this age (20x magnification).



(b) Immunoexpression of Neurturin in testis from an adult control animal. As with the immunoexpression of the GFR- α 2 receptor at this age, intense staining of occasional B-spermatogonia can be visualised (solid arrows) (10x magnification).



also observed within the testes at other ages. In the neonatal period immunoexpression of both GDNF and Neurturin is observed in a proportion of the germ cells (Figures 5.5a and 5.6a). Although there is some interstitial staining for Neurturin, this is not as widespread as that observed for the GFR- α 2 receptor at this age. However, the pattern of immunostaining for these ligands in adult testes did reflect that seen with the GFR- α 2 receptor at this age (Figures 5.5b and 5.6b).

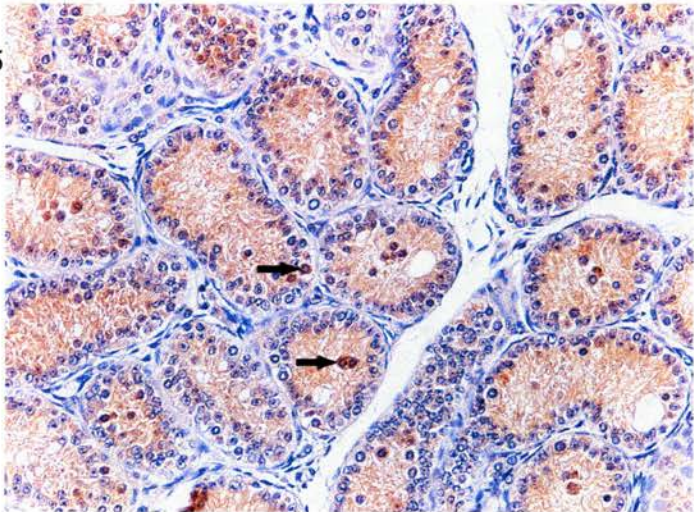
5.3.3 Other Growth Factor Receptors

The prominent immunoexpression in prepubertal germ cells of the receptors discussed above was not observed with other growth factor receptors investigated.

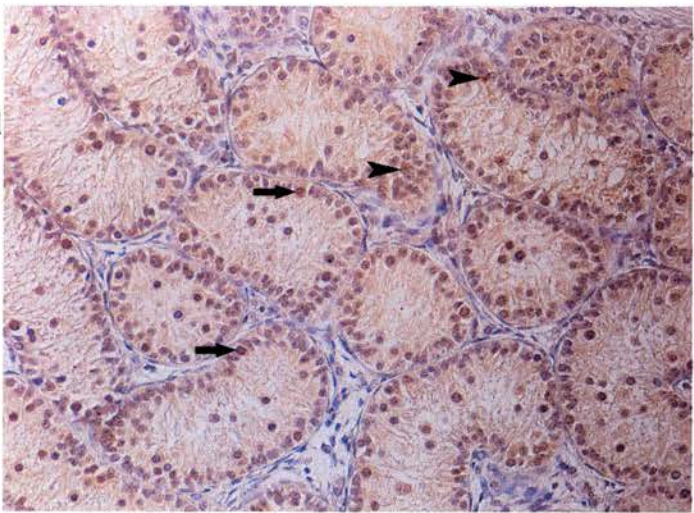
Prepubertally, very few germ cells expressed the IGF-1 receptor (Fig 5.7a), although problems with background staining prevented definitive interpretation of this result. Immunoexpression of the FGF receptor was noted in both germ cells and Sertoli cells at 35 weeks of age (Fig 5.7b), although artefactual staining was also present and thus reliable conclusions could not be drawn. However, expression of FGF-4 was also noted in both germ cells and Sertoli cells at this age (Fig 5.7c), which would correlate with these findings. Immunoexpression of the GM-CSF receptor was not observed at all within the testes at any age (Fig 5.7d), although the specificity of the antibody used was uncertain as marmoset thymus, used as a positive control, was also negative.

Figure 5.7 Immunoexpression within 35-week-old marmoset testis of (a) IGF-1 receptor (b) FGF receptor (c) FGF-4 ligand (d) GM-CSF receptor

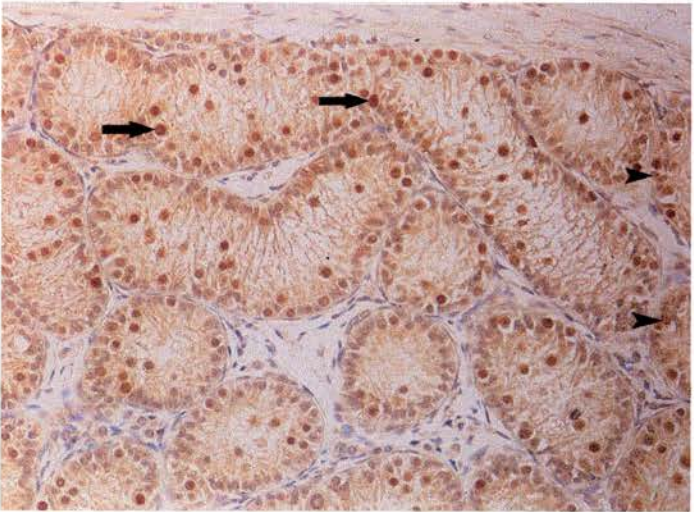
(a) Immunoexpression of the IGF-1 receptor in testis from a 35 week old control animal. Occasional germ cells are immunopositive for this receptor (solid arrows), although background staining in the majority of sections analysed prevented definitive analysis (20x magnification).



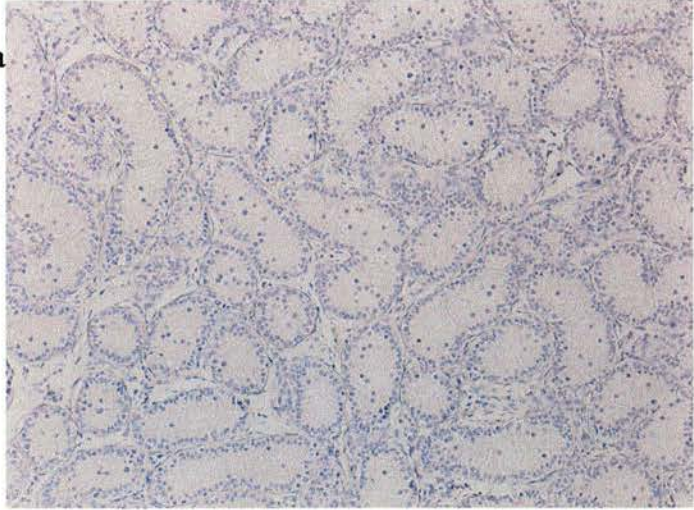
(b) Immunoexpression of the FGF-receptor in testis from a 35 week old control animal. Both immunopositive germ cells (solid arrows) and Sertoli cells (arrowheads) are noted, although staining is not clear and conclusions are therefore difficult to draw (20x magnification).



(c) Immunoexpression of FGF-4 ligand in testis from a 35 week old control animal. As suggested with the FGF-receptor, immunopositive germ cells (solid arrows) and Sertoli cells (arrowheads) are noted, although similarly staining is not clear (20x magnification).



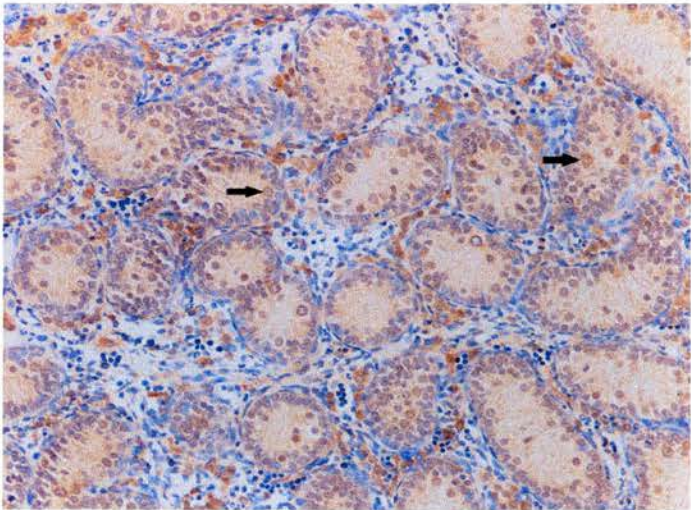
(d) Immunoexpression of the GM-CSF receptor in testis from a 35 week old control animal. No staining is observed at all, which may represent lack of expression of this receptor in this tissue or a technical problem relating to the antibody used (10x magnification).



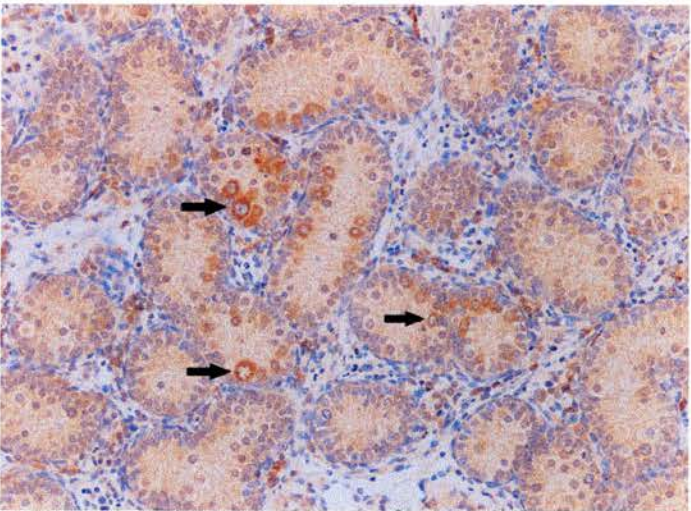
Immunoexpression of the LIF receptor was noted in occasional germ cells within the neonatal period (Fig 5.8a), and this appeared more prominent in testes from animals that had received previous treatment with a GnRH antagonist (Fig 5.8b). Expression of this receptor was also observed within interstitial cells. Immunoexpression in germ cells was not observed beyond early infancy, although the interstitial cell staining persisted (Fig 5.8c and d).

Figure 5.8 Immunoexpression of the LIF Receptor within the marmoset testis (a) 4-week-old neonate (b) 4-week-old neonate with suppressed neonatal testosterone surge (c) 35-week-old infant (d) Adult

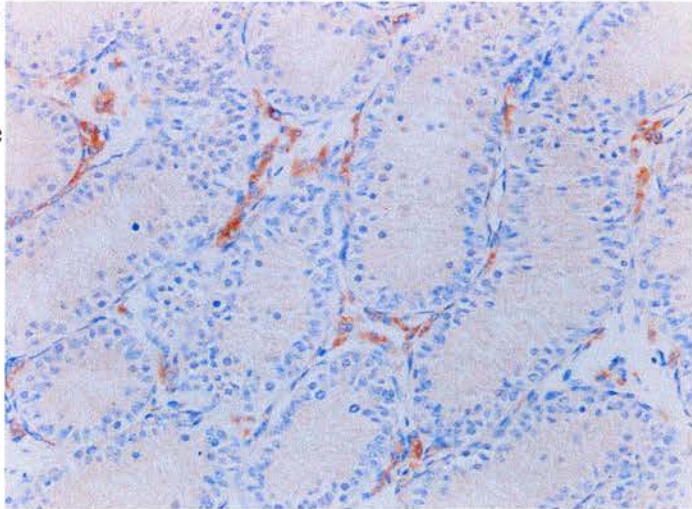
(a) Immunoexpression of the LIF receptor in testis from a 4 week old control animal. Occasional germ cells are immunopositive for this receptor (solid arrows). Staining is also noted within the interstitium (20x magnification).



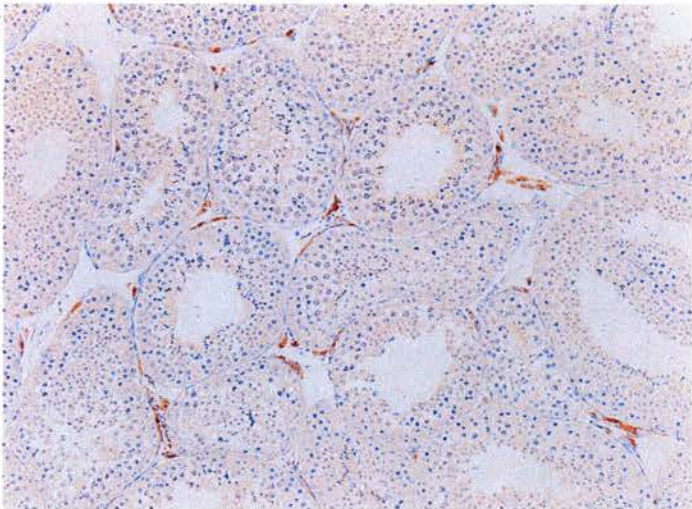
(b) Immunoexpression of the LIF receptor in testis from the 4 week old co-twin of the above animal, which has received treatment with a GnRH antagonist since shortly after birth. Several germ cells are immunopositive for this receptor (solid arrows), and this appears more pronounced than that in the control animal (20x magnification).



(c) Immunoexpression of the LIF receptor in testis from a 35 week old control animal. Although interstitial staining is noted, there are no immunopositive cells within the seminiferous tubule (20x magnification).



(d) Immunoexpression of the LIF receptor in testis from an adult control animal. In a similar pattern to that observed at 35 weeks of age, interstitial staining is present but no cells within the seminiferous tubule are immunopositive (10x magnification).



5.3.4 Activin Receptor

The activin receptor IIA was localised to both the nucleus and cytoplasm of germ cells. At 35 weeks of age, the majority of germ cells within the testis express this receptor, and this pattern is unaffected by previous treatment with a GnRH antagonist (Fig 5.9).

5.3.5 Confocal Microscopy

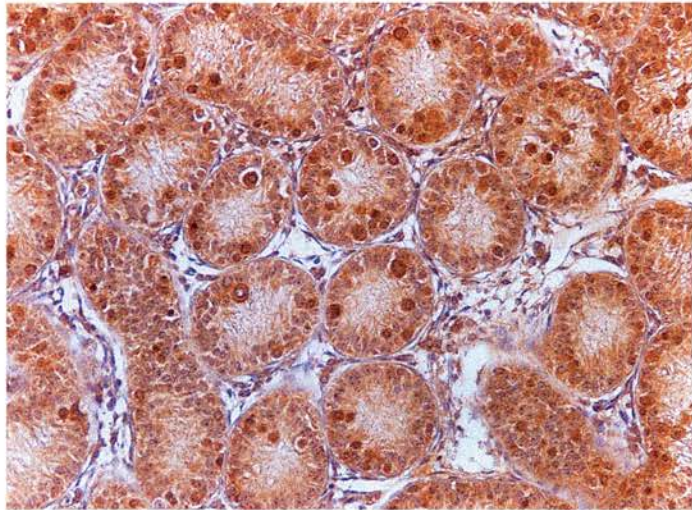
From the growth factor receptors studied, the EGF and the GFR- α 2 receptors were investigated further as both were localized to a proportion of germ cells prepubertally. It was hypothesised that the population of germ cells expressing the EGF or GFR- α 2 receptors would be those that were actively proliferating. However, the opposite was found, as PCNA immunopositive germ cells did not co-express either growth factor receptor (Fig 5.10). This pattern of expression was unaffected by previous treatment with a GnRH antagonist.

With regard to the EGF receptor, which is widely expressed outside of the prepubertal phase of development, germ cells expressing this receptor were also negative for PCNA within the neonatal period (Fig 5.11a). However, during adulthood, some germ cells expressing the EGF receptor were PCNA positive (Fig 5.11b).

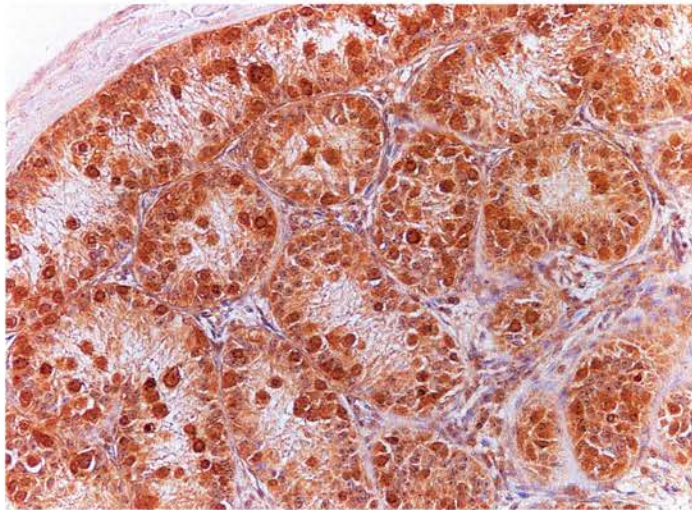
Confocal microscopy for these two growth factor receptors was also attempted in combination with either Ki67 or Phosphohistone H3. However, as mentioned previously, the heat-induced antigen retrieval step required for the detection of these proliferation markers affects the quality of detection of the cytoplasmic growth factor receptors. Despite this, the actively proliferating cells still appeared to be separate from the population of germ cells expressing either growth factor receptor (Fig 5.12).

Figure 5.9 Immunoexpression of the Activin Receptor IIA in marmoset testis
(a) 35-week-old infant (b) 35-week-old co-twin treated prepubertally with a
GnRH antagonist (c) Negative control slide

(a) Immunoexpression of the activin receptor IIA in testis from a 35 week old control animal. A large proportion of germ cells are immunopositive for this receptor (20x magnification).



(b) Immunoexpression of the activin receptor IIA in testis from the 35 week old co-twin of the above animal, which has received prior treatment with a GnRH antagonist. A similar pattern of expression exists, with a large proportion of immunopositive germ cells observed (20x magnification).



(c) Section of testis from a 13 week old control animal, which has been treated identically to the above sections other than the omission of the primary antibody, in order to act as a negative immunohistochemical control (20x magnification). No expression is observed, whereas sections at this age with primary antibody included were positive.

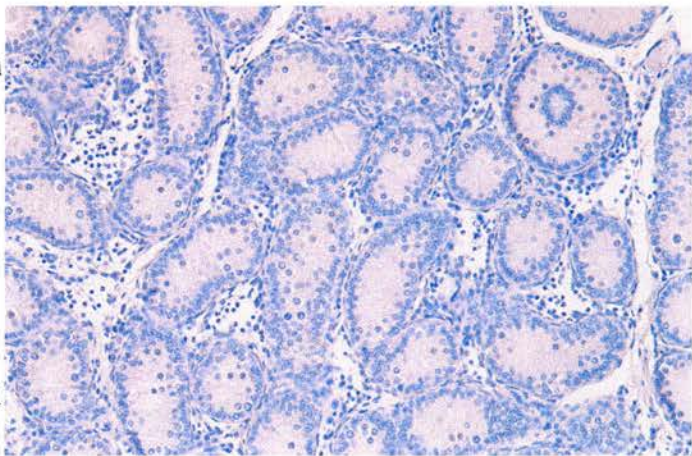
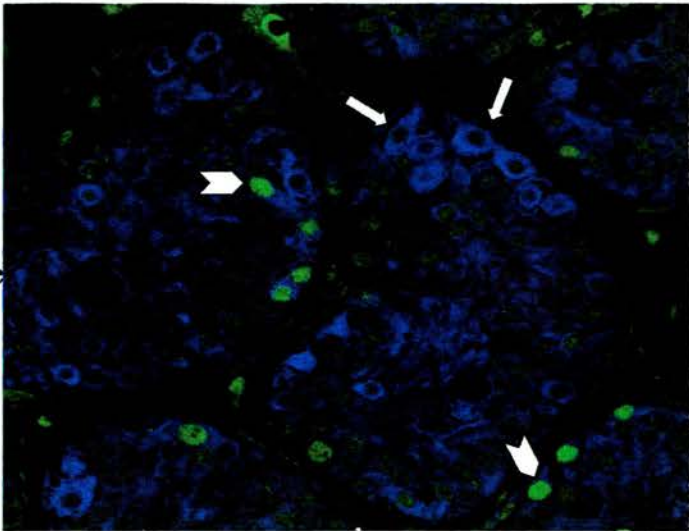


Figure 5.10 Localisation of the proliferation marker PCNA (green) in the 35-week-old marmoset testis with either, (a) the EGF receptor (blue) or (b) GFR- α 2 (blue) using confocal microscopy

(a) Immunoexpression of PCNA (green) and the EGF receptor (blue) in testis from a 35 week old control animal, using confocal microscopy. PCNA is localised to the nucleus of proliferating cells (indicated by arrowheads). The EGF receptor is expressed in the cytoplasm of a proportion of germ cells (solid arrows). There is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time.



(b) Immunoexpression of PCNA (green) and the GFR- α 2 receptor (blue) in testis from a 35 week old control animal, using confocal microscopy. PCNA is localised to the nucleus of proliferating cells (indicated by arrowheads). The GFR- α 2 receptor is expressed in the cytoplasm of a proportion of germ cells (solid arrows). As with the pattern of expression of the EGF receptor, there is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time.

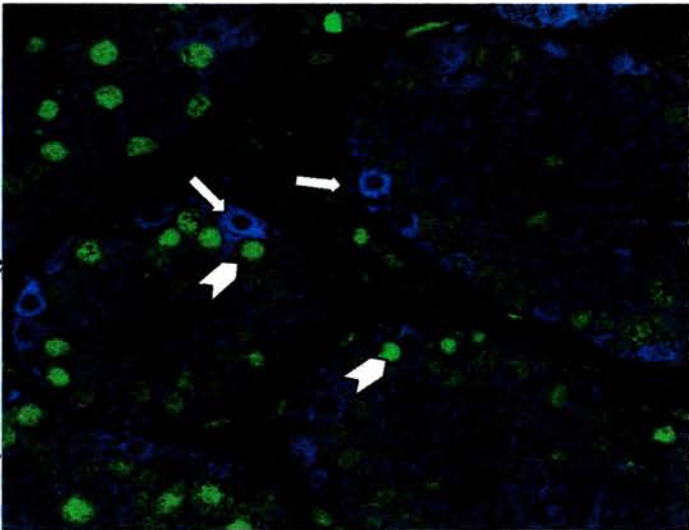
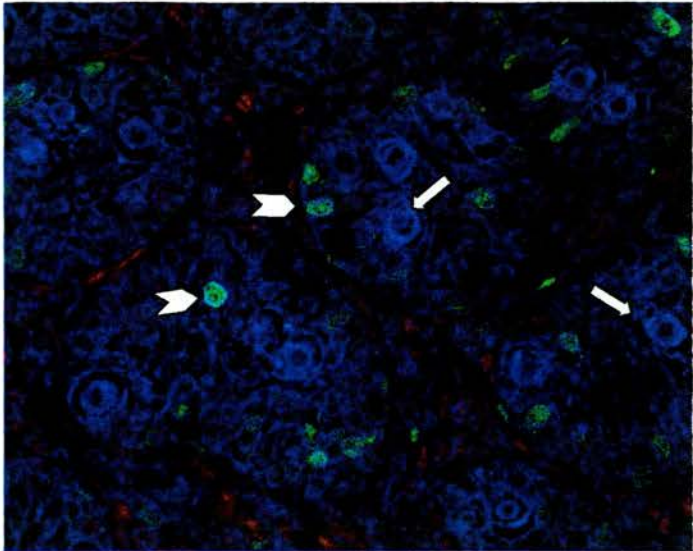


Figure 5.11 Localisation of the proliferation marker PCNA (green) with the EGF receptor (blue) in the marmoset testis using confocal microscopy (a) 7 week old (b) adult

(red staining represents counterstain with propidium iodide)

(a) Immunoexpression of PCNA (green) and the EGF receptor (blue) in testis from a 7 week old control animal, using confocal microscopy. PCNA is localised to the nucleus of proliferating cells (indicated by arrowheads). The EGF receptor is expressed in the cytoplasm of a proportion of germ cells (solid arrows). As with immunoexpression of these antigens at 35 weeks of age, there is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time.



(b) Immunoexpression of PCNA (green) and the EGF receptor (blue) in testis from an adult control animal, using confocal microscopy. As before, PCNA is localised to the nucleus of proliferating cells and the EGF receptor is expressed in the cytoplasm of a proportion of germ cells. However, unlike at earlier ages, occasional germ cells expressing the EGF receptor are actively proliferating (indicated by solid arrows).

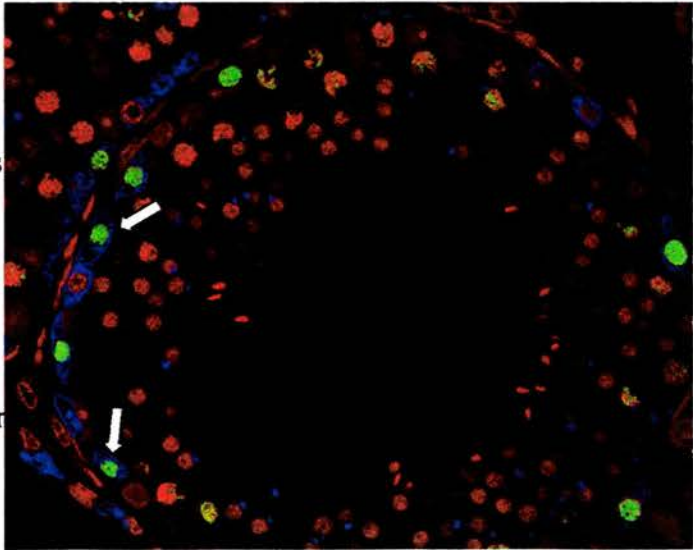
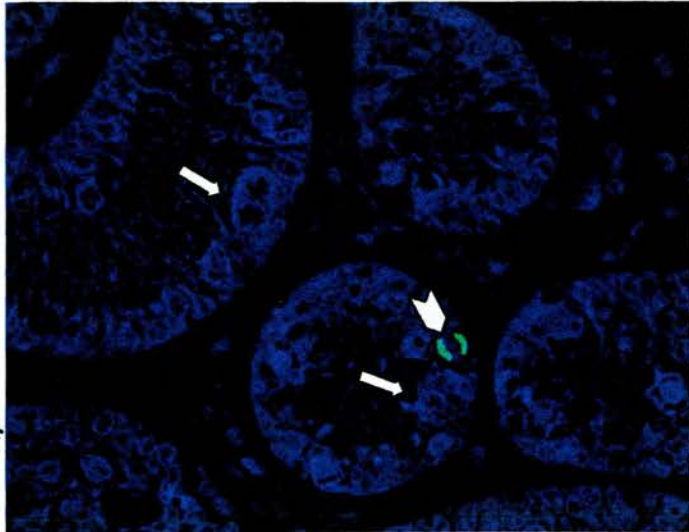
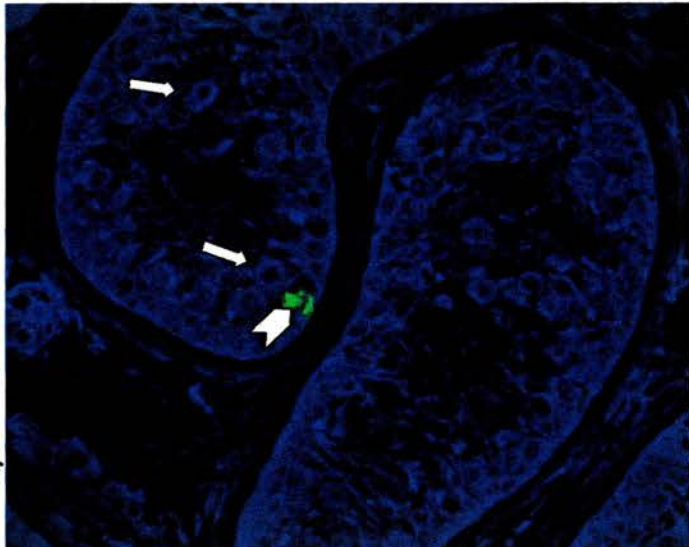


Figure 5.12 Localisation of further proliferation markers (green) in the 35-week-old marmoset testis with the growth factor receptors (blue) using confocal microscopy. (a) Phosphohistone H3 and the EGF receptor (b) Phosphohistone H3 and GFR- α 2 (c) Ki67 and GFR- α 2

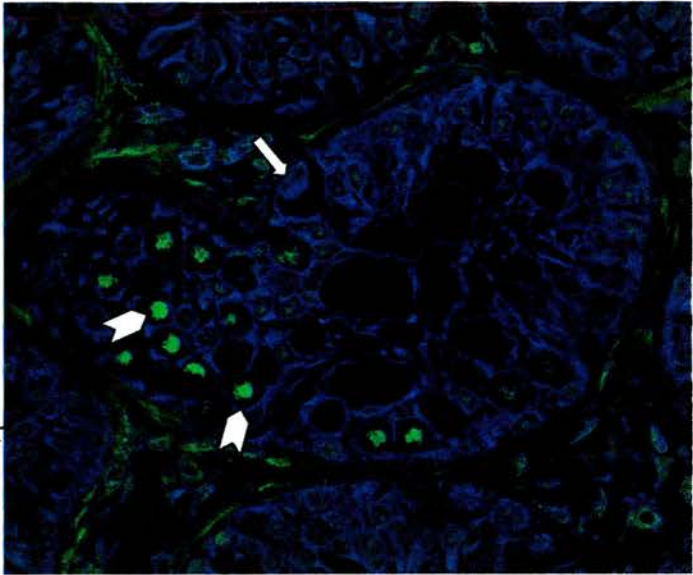
(a) Immunoexpression of Phosphohistone H3 (green) and the EGF receptor (blue) in testis from a 35 week old control animal, using confocal microscopy. Phosphohistone H3 is localised to the nucleus of a proliferating cell within the field (indicated by arrowhead). As before, the EGF receptor is expressed in the cytoplasm of a proportion of germ cells (solid arrows). However, the quality of immunostaining for the EGF receptor is impaired due to the antigen retrieval process. Despite this, it appears that these antigens are not co-localised.



(b) Immunoexpression of Phosphohistone H3 (green) and the GFR- α 2 receptor (blue) in testis from a 35 week old control animal, using confocal microscopy. Phosphohistone H3 is localised to the nucleus of a proliferating cell within the field (indicated by arrowhead). As before, the GFR- α 2 receptor is expressed in the cytoplasm of a proportion of germ cells (solid arrows). However, the quality of immunostaining for the GFR- α 2 receptor is impaired due to the antigen retrieval process. Despite this, it appears that these antigens are not co-localised.



(c) Immunoexpression of Ki67 (green) and the GFR- α 2 receptor (blue) in testis from a 35 week old control animal, using confocal microscopy. Ki67 is localised to the nucleus of proliferating cells (indicated by arrowheads). As before, the GFR- α 2 receptor is expressed in the cytoplasm of a proportion of germ cells (such as that indicated by the solid arrow). However, the quality of immunostaining for the GFR- α 2 receptor is impaired due to the antigen retrieval process. Despite this, it appears that these antigens are not co-localised.



5.4 Discussion

The aim of this part of the study was to identify factors that potentially act to regulate spermatogonial proliferation in the prepubertal marmoset testis. Knowledge of this process would not only improve our understanding of the physiology of spermatogenesis and testis development, but would also aid in devising strategies to protect fertility in boys treated for cancer.

This study has identified a number of growth factor receptors that are prominently expressed by a proportion of germ cells, particularly during prepubertal life. The role these growth factors may have within the testis is uncertain at present, but the presence of these receptors warrants further investigation.

5.4.1 Epidermal Growth Factor

The EGF receptor is a transmembrane tyrosine kinase receptor (Ushiro and Cohen, 1980) for members of the transforming growth factor family, including EGF and Transforming Growth Factor- α (TGF- α). This family of growth factors are involved in cellular proliferation and differentiation in many tissues.

Within the testis, EGF and related ligands have been shown to have a role in the regulation of embryonic testicular growth in the rat (Levine *et al*, 2000). In adult mice, removal of the submandibular gland, the predominant source of EGF, results in infertility, which is reversed by EGF administration (Liu *et al*, 1994). In addition, proliferation of both murine germ stem cells (Kanatsu-Shinohara *et al*, 2003) and rat spermatogonia (Wahab-Wahlgren *et al*, 2003) is stimulated *in vitro* by culture medium containing EGF.

However, overexpression of EGF in transgenic mice also results in infertility, with decreased production of spermatids and spermatozoa (Wong *et al*, 2000). It has therefore been suggested that EGF is required at a specific concentration to support spermatogenesis, in particular for progress from meiosis I to meiosis II (Wong *et al*, 2000). *In vitro* studies using adult mouse cryptorchid testes lend support to this theory, in that EGF in a range of concentrations exhibits differential effects on proliferation and differentiation of type A spermatogonia (Haneji *et al*, 1991). Indeed, this latter study suggested EGF results predominantly in inhibition of the proliferation and differentiation of type A spermatogonia, perhaps by blocking the action of FSH. Thus uncertainties persist regarding the precise role of EGF within the mammalian testis.

The EGF receptor has been previously localized within the testis in a number of animal models. In the prepubertal boar testis weak staining of both spermatogonia and somatic cells has been described (Caussanel *et al*, 1996). More mature germ cells demonstrated increased staining intensity in both the prepubertal animal and the adult, which in the latter was noted particularly during meiosis and spermiogenesis.

Previous studies using primates have localized the EGF receptor to Leydig cells, Sertoli cells and peritubular cells, but have not demonstrated germ cell staining (Radhakrishnan and Suarez-Quian, 1992). Foresta and Varotto (1994) investigated human testicular tissue, obtained via fine needle aspiration from infertile men, and demonstrated weak immunostaining of the EGF receptor in Sertoli and germ cells. Interestingly, the intensity of this staining increased in patients with higher FSH levels.

The results presented here are the first to demonstrate such prominent immunostaining of the EGF receptor in prepubertal germ cells in a primate model. The receptor was localized to a proportion of germ cells, the majority of which were identified as B spermatogonia. In contrast to previous studies, the receptor was not detected on somatic cells.

Although, as mentioned previously, the submandibular gland is the predominant source of circulating EGF in mice, Leydig cells are the principal source of EGF in the testis (Yan *et al*, 1998). It therefore seems likely that local production of EGF plays a role in the paracrine regulation of germ cell proliferation. The identification of the EGF receptor on numerous germ cells supports this hypothesis, although the precise role of EGF is yet to be elucidated.

Co-expression of the EGF receptor with markers of proliferation using immunofluorescence demonstrated that those germ cells expressing this growth factor receptor were quite separate from the actively proliferating cells. This finding was unexpected as it was suspected that EGF would stimulate spermatogonial proliferation. This could suggest that EGF is not involved with the regulation of this process at all. However, because these populations of germ cells appeared quite distinct, in that no germ cell identified as proliferating was also expressing the EGF receptor, EGF may actually play an *inhibitory* role in spermatogonial proliferation during prepubertal life. This could be via inhibition of intrinsic germ cell proliferation or, alternatively, may occur secondary to interference of stimulatory pathways. An inhibitory action of EGF within the testis has been suggested in rodent

models, as discussed previously (Wong *et al*, 2000; Haneji *et al*, 1991). The results presented here suggest a similar role in the prepubertal primate testis.

Both FSH and testosterone stimulate the mRNA expression of the EGF receptor in the perinatal rat testis (Cupp and Skinner, 2001). In addition, EGF secretion is increased by androgenic stimulation (Cain *et al*, 1994). Thus the hypothalamic-pituitary-gonadotrophin axis does influence EGF and its actions. The immunoexpression of the EGF receptor within 35-week-old marmoset testis presented here appeared to be more intense in animals treated prepubertally with a GnRH antagonist. As FSH levels will be low in these animals this would appear to contrast with the above studies.

However, this finding was not consistent amongst all co-twin pairs and was not quantified; therefore firm conclusions are difficult to draw. Further work is required to elucidate both the regulation of EGF and its role within the testis, particularly with regards to spermatogonial proliferation, and it is hoped that the marmoset model will yield further information.

5.4.2 Glial Cell Line-Derived Neurotrophic Factor Family

The GFR- α 2 receptor is a receptor for members of the GDNF family of neurotrophic factors, which act to promote the survival of various peripheral and central neurons. This family consists of GDNF itself, along with neurturin, persephin and artemin, and are distant members of the transforming growth factor- β superfamily (Saarma, 2000). These factors act via a receptor complex consisting of one of four co-receptor subunits (GFR α 1-4) as a ligand-binding component, together with a common receptor tyrosine kinase (RET) as a signalling component (Takahashi, 2001). In general, GDNF binds to GFR- α 1, neurturin to GFR- α 2, persephin to GFR- α 3 and artemin to

GFR- α 4, although some cross-reactivity is felt to occur, at least *in vitro* (Wang *et al*, 2000).

In addition to their role within the nervous system, GDNF, and to a lesser extent other family members, are involved in renal development, particularly with regards to ureteric budding and branching (Pichel *et al*, 1996).

Expression of GDNF mRNA has been demonstrated in Sertoli cells (Trupp *et al*, 1995), suggesting this factor may also be involved within the testis. Using mouse models, Meng *et al* (2000) have implicated GDNF as having a role in the regulation of spermatogenesis. Gene-targeted mice with reduced GDNF demonstrate depletion of germ stem cell reserves, whereas mice over-expressing this factor have an accumulation of undifferentiated spermatogonia, secondary to a differentiation block. The authors suggest that stem spermatogonia favour differentiation when GDNF levels are low, and favour self-renewal when levels are high. Over-expression of neurturin in mice models also affects spermatogenesis, although only transiently and predominantly affects spermatocytes and spermatids (Meng *et al*, 2001a).

Expression of both GFR- α 1 and GFR- α 2 has been previously demonstrated in testicular germ cells in the mouse, and GDNF and neurturin are present in Sertoli cells in this species (Viglietto *et al*, 2000). These ligands stimulate DNA synthesis in spermatogonia. In addition, murine spermatogonial stem cells proliferate in culture medium containing GDNF (Kanatsu-Shinohara *et al*, 2003).

Based on these previous studies it seems likely that local production of GDNF and, perhaps to a lesser extent, neurturin, have a role in the paracrine regulation of spermatogonial proliferation and differentiation. The results in the marmoset presented here support this hypothesis, with prominent immunoexpression of the GFR- α 2 receptor demonstrated in a significant proportion of germ cells in the prepubertal primate testis. However, the expression of this receptor was far less prominent outside this period of testicular development. In the neonatal period expression was noted within the interstitium only, and in postpubertal animals the receptor was localized to a very small number of basal germ cells. Regulation of germ cell proliferation involving GFR- α 2 may therefore be particularly important in the prepubertal primate but perhaps involves a smaller population of germ cells at other ages. In addition, these results implicate neurturin as having a potentially more important role than GDNF; although as discussed cross-reactivity of the receptors does occur.

Indeed, expression of the GFR- α 1 receptor, reported to be the main receptor for GDNF, was demonstrated at all ages but was limited to a very small number of germ cells, as discussed fully in chapter 6. The cells expressing this receptor could include germ stem cells themselves, supporting a role for GDNF in the regulation of the fate of stem spermatogonia, as suggested by Meng *et al* (2000).

Immunolocalization of both GDNF and neurturin detected the presence of these ligands in Sertoli cells and germ cells in marmoset testes. Production of GDNF by Sertoli cells has been previously reported, as discussed above, and is presumed to represent paracrine involvement of these factors within the testis. However, the

expression of these ligands on germ cells suggests autocrine regulation may also play a role.

It has been demonstrated that FSH can stimulate the production of GDNF from Sertoli cells, and this in turn increases proliferation of undifferentiated spermatogonia (Tadokoro *et al*, 2002). However, this study involved murine models with testes in which germ stem cells proliferate but do not differentiate, and are therefore unable to produce offspring. FSH levels within this model are increased beyond physiological levels, and the authors postulate that this GDNF/FSH pathway is an important regulatory mechanism in the recovery from impaired spermatogenesis, rather than in normal states. Within the study reported here, immunoexpression of GFR- α 2 was unaffected by prior treatment with a GnRH antagonist, although FSH levels will be suppressed rather than raised following this treatment.

As with the studies investigating the EGF receptor, attempts to co-localize GFR- α 2 with markers of proliferation suggest that germ cells expressing this growth factor receptor are quite separate from those that are actively proliferating. This raises the possibility that the role of the GDNF family in spermatogonial proliferation is inhibitory, at least in the prepubertal primate. This appears to contrast with the published data using mouse models discussed above, and again illustrates the difficulty of comparing rodent and primate models to study spermatogenesis. It is hoped that further investigation using the marmoset model will shed more light into the role of these factors.

5.4.3 Leukaemia Inhibitory Factor

LIF is a member of the interleukin-6 cytokine family and, in addition to effects on the haematopoietic system, acts to modulate endocrine function in a variety of tissues, acting via a common LIF receptor (Auernhammer and Melmed, 2000). Within the reproductive system, LIF plays an important role in blastocyst implantation and the establishment of pregnancy (Lass *et al*, 2001). With regards to the male reproductive system, the addition of LIF to cell cultures from the neonatal rat testis results in enhanced survival of both Sertoli cells and gonocytes (de Miguel *et al*, 1996). Rather than an effect on cell proliferation, the mechanism of action is felt to be via prevention of apoptosis (Pesce *et al*, 1993). More recently it has been demonstrated that LIF also inhibits steroidogenesis in the Leydig cell (Manduit *et al*, 2001). In addition, germ stem cells from the mouse proliferate in culture medium containing a number of growth factors including LIF (Kanatsu-Shinohara *et al*, 2003).

Therefore, as with other cytokines and growth factors, LIF is likely to act within the testes in a paracrine or autocrine manner, and perhaps has a particular role in regulation of the germ cell population during the perinatal period of testicular development. Peritubular cells appear to be the principal source of LIF (Piquet-Pellorce *et al*, 2000), and using porcine models, the LIF receptor has been demonstrated on Leydig cells, Sertoli cells and spermatogonia (Manduit *et al*, 2001).

Using the marmoset model here, immunoexpression of the LIF receptor in Leydig cells, from the neonatal period through to adulthood, has been demonstrated. With regards to germ cell staining, prominent expression of this receptor on a number of germ cells during the neonatal phase of testicular development has been shown.

However, this does not persist beyond around 18-24 weeks of age. This certainly supports the hypothesis that LIF acts to regulate the germ cell population during early life, and further studies are warranted to investigate its precise role. Interestingly, immunoexpression of the LIF receptor in germ cells appeared more pronounced in animals that had had their neonatal testosterone surge suppressed by prior administration of a GnRH antagonist. One could speculate that increased receptor expression may increase survival of these germ cells via a reduction in apoptosis, and that this is perhaps a compensatory mechanism of the germ cell, secondary to reduced Sertoli cell support due to lowered FSH levels.

The lack of expression of the LIF receptor in germ cells from animals at older ages suggests that this cytokine is perhaps less important in germ cell regulation during the 'infancy' phase of testicular development, and is therefore unlikely to contribute to the development of a strategy to protect fertility in boys treated for cancer.

5.4.4 Fibroblast Growth Factor

FGF represents a further family of growth factors that have been previously implicated in the regulation of spermatogenesis within the mammalian testis. This family consists of 22 ligands, which are known to act via four tyrosine kinase receptors (Bellosta *et al*, 2001), and they play key roles in embryonic development. In adult tissues, FGF members are thought to be involved in angiogenesis and wound healing. More recently, expression of the gene encoding FGF-4 has been demonstrated in Sertoli cells of the adult testis, and over-expression of this gene in transgenic mice results in markedly enhanced spermatogenesis (Yamamoto *et al*, 2002). Furthermore, these transgenic mice demonstrated improved recovery

following adriamycin-induced testicular toxicity, although the precise mechanism underlying this remains uncertain.

FGF receptors have been demonstrated on germ cells and somatic cells of the testes, both in the immature rat (Cancilla and Risbridger, 1998) and adult men (Steger *et al*, 1998). In addition, murine stem spermatogonia have been cultured in medium containing basic FGF (Kanatsu-Shinohara *et al*, 2003).

Using the marmoset model we detected immunoexpression of the FGF-1 receptor within the testis, in both germ cells and Sertoli cells, although artefactual staining affected the interpretation of these results and, as such, conclusions regarding functional significance are difficult to draw. Investigation of FGF-4, as a potential ligand involved in spermatogenesis, detected its presence in both germ cells and Sertoli cells, correlating with the data obtained from rodent models. Thus members of the FGF family may play a role in the regulation of spermatogenesis in the primate, although further investigation is required.

5.4.5 Insulin-like Growth Factor

Similarly, conclusions regarding the role of IGF-1 in the prepubertal primate testis are difficult to draw based on this immunohistochemical study. This growth factor has a variety of functions within a number of tissues. With regard to the testes, differentiation of murine A spermatogonia can be induced by culture medium containing IGF-1 (Tajima *et al*, 1995), and IGF-1 has been shown to stimulate DNA synthesis in rat spermatogonia *in vitro* (Soder *et al*, 1992). In addition, studies of

IGF-1 levels in seminal plasma from infertile men have also implicated this growth factor as having a role in spermatogenesis (Colombo and Naz, 1999).

Although immunoexpression of the IGF-1 receptor was detected in the prepubertal marmoset, this was only in a minority of germ cells and problems with background staining affected interpretation of these results. It should be noted, however, that because only a small number of germ cells express a receptor, this does not exclude a significant role of the corresponding ligand. This is particularly true if those germ cells with positive expression are actually stem cells, as described in the following chapter. However, the small number of positive cells does make interpretation and further analysis more problematic.

5.4.6 Granulocyte Macrophage Colony Stimulating Factor

Immunoexpression of the receptor for GM-CSF was not detected in the marmoset testis at any age. GM-CSF is a cytokine that stimulates the proliferation and maturation of haematopoietic cells (Gasson, 1991). However, it is also produced by testicular macrophages in high concentrations (Kern *et al*, 1995) and can cross the blood-testis barrier in mice (McLay *et al*, 1997). Dirami *et al* (1999) have demonstrated an enhanced survival of porcine spermatogonia in culture medium containing GM-CSF, although the effect of this growth factor on proliferation was not established. In addition, the authors report localization of the GM-CSF receptor in type A spermatogonia in the mouse. More recently, the presence of this receptor has been demonstrated in germ cells from adult men and in bull spermatozoa (Zambrano *et al*, 2001). The addition of GM-CSF to these latter cells resulted in an increased

uptake of both glucose and vitamin C, thus suggesting a role for this ligand in spermatozoal physiology.

The lack of expression of the GM-CSF receptor in the marmoset may simply reflect that this receptor is not present within these testes. However, given the previous studies discussed above, and the lack of expression in marmoset thymus as a positive control, it seems more likely that there was a problem with the primary antibody used, and that further investigation using alternative antibodies is required before firm conclusions are drawn.

5.4.7 Activin

Activins, along with inhibins, are members of the transforming growth factor- β family, and are involved in the regulation of FSH secretion (de Kretser *et al*, 2001). However, it has also been suggested that these factors may have a local role within the testis (Mather *et al*, 1997). Activin has been shown to stimulate spermatogonial proliferation *in vitro*, in co-cultures of rat germ cells with Sertoli cells (Mather *et al*, 1990). In addition, activin has been shown to regulate gonocyte and Sertoli cell proliferation in the postnatal rat testis (Boitani *et al*, 1995; Meehan *et al*, 2000).

Activins signal via the type II receptor serine kinases, ActRIIA or ActRIIB (Massague, 1998). These receptors are expressed by Sertoli cells, spermatocytes and round spermatids in the rat (de Winter *et al*, 1992). Expression of the ActRIIA receptor has also been detected in gonocytes and interstitial cells of the human foetal testis (Anderson *et al*, 2002).

Within the prepubertal marmoset testis, a high proportion of germ cells have been shown to express the ActRIIA receptor, a pattern that appeared unaffected by prior treatment with a GnRH antagonist. The presence of this receptor may support a paracrine role of activin within marmoset testis. However, the high level of expression throughout many stages of germ cell development suggests activin is unlikely to become a realistic target to suppress spermatogonial proliferation, and thus protect fertility in boys treated for cancer.

This part of the study has identified a number of growth factor receptors within the prepubertal marmoset testis. These receptors demonstrate particular patterns of expression, and this information will contribute to our understanding of the role these factors may have within the testis and, in particular, in the regulation of spermatogenesis.

Chapter 6

Results- Stem Cell Markers

6.1 Introduction

It is hoped that the identification of growth factors that may act to regulate spermatogonial proliferation prepubertally, as discussed in chapter 5, will lead to the development of strategies to protect fertility in boys treated for cancer. However, as survival of stem spermatogonia is essential for future fertility, this study also investigated the immunoexpression of putative stem cell markers within the testis, using this marmoset model. Stem spermatogonia are pluripotent germ cells capable of both self-renewal and differentiation into other germ cell types. The identification of stem spermatogonia would subsequently allow analysis of their relationship with some of the growth factor receptors identified previously.

6.2 Methods

To identify stem spermatogonia within the marmoset testis, the immunoexpression of markers reported to be unique to such cells was examined. Should it be possible to reliably identify stem spermatogonia, the intention was to co-localise these cells, both with markers of cell proliferation and the growth factor receptors discussed in chapter 5.

6.2.1 Animals

Sections of testis taken from marmosets killed at various time points, from the neonatal period to adulthood, were investigated. Animals used were as detailed previously.

6.2.2 Immunohistochemical Markers

Potential stem cell markers were selected on the basis of rodent models that have previously investigated this cell population. C-kit is the trans-membrane receptor for Stem Cell Factor (SCF) and is expressed in neonatal gonocytes in the rat (Orth *et al*, 1996). Oct-4 is a transcription factor expressed in totipotent embryonic stem and germ cells in the mouse (Pesce and Scholer, 2001), and has been used for the immunohistochemical detection of pluripotent cells in human germ cell tumours (Looijenga *et al*, 2003). GFR- α 1, the receptor for GDNF, is expressed in murine spermatogonia up to the A aligned stage (Meng *et al*, 2000).

6.2.3 Immunohistochemistry

The immunohistochemical methods used for detecting the expression of these markers are detailed in chapter 3. As with previous antibodies studied, each one required trials under differing conditions to establish the optimal method of detection.

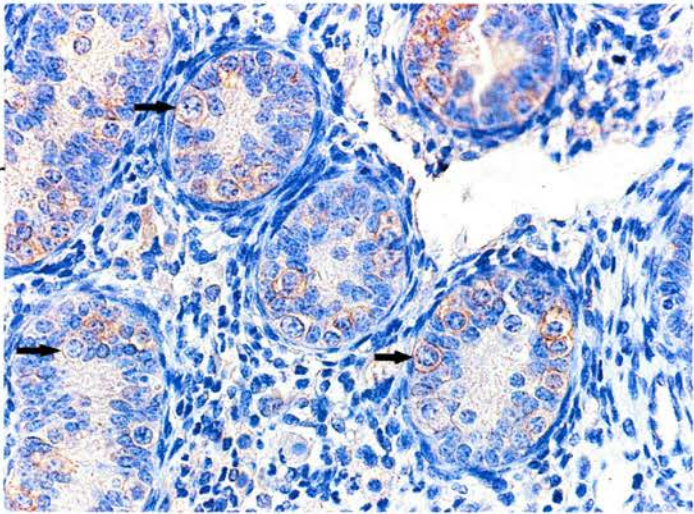
Co-localisation studies were initially performed using immunofluorescence. However, immunopositive cells were difficult to identify with this method due to their small number, together with problems of background staining. Therefore, double staining was also performed using conventional immunohistochemistry. Whilst one antibody was visualised with DAB, the second antibody was visualised with Fast Blue, as detailed in section 3.5.

6.3 Results

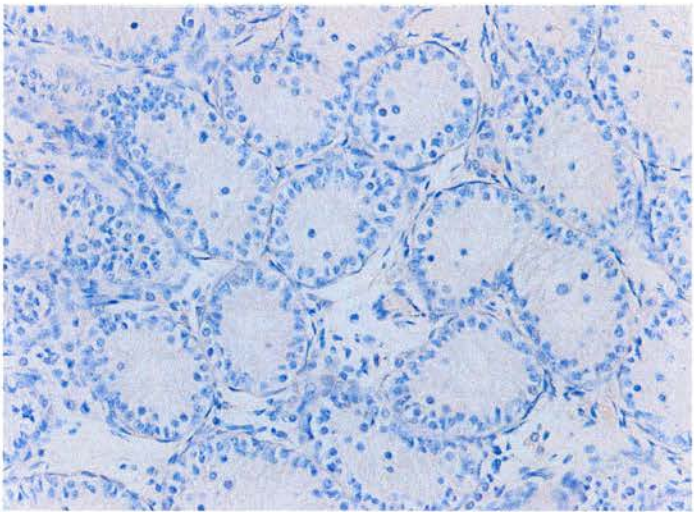
C-kit was localized to the peri-membranous region of germ cells within the neonatal period (Fig 6.1a). However, expression within the prepubertal age group was not

Figure 6.1 Immunoexpression of C-kit within (a) 1-day-old neonatal marmoset testis (b) 35-week-old marmoset testis (c) Adult marmoset testis

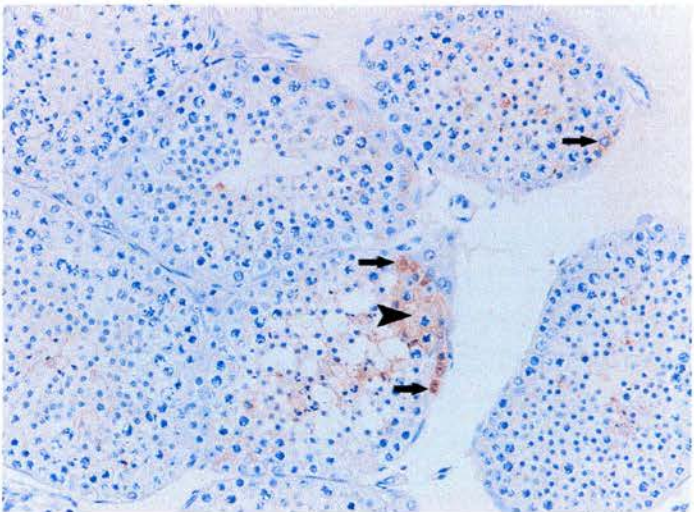
(a) Immunoexpression of C-kit in testis from a 1 day old control animal. Peri-membranous expression of this antigen is observed in a number of germ cells at this age (arrowed) (40x magnification).



(b) Immunoexpression of C-kit in testis from a 35-week-old control animal. No expression of this antigen is observed at this age (20x magnification).



(c) Immunoexpression of C-kit in testis from an adult control animal. Occasional germ cells are immunopositive for this antigen (arrowed), although some non-specific staining exists (indicated by arrowhead), making definitive interpretation difficult (20x magnification).



observed (Fig 6.1b). Limited expression within germ cells was noted in adulthood, although apparent non-specific staining affected interpretation at this age (Fig 6.1c).

Expression of Oct-4 within germ cell nuclei was also prominent in the neonatal period, but staining in older ages was rather non-specific, in that large numbers of both germ cells and Sertoli cells appeared labelled (Fig 6.2).

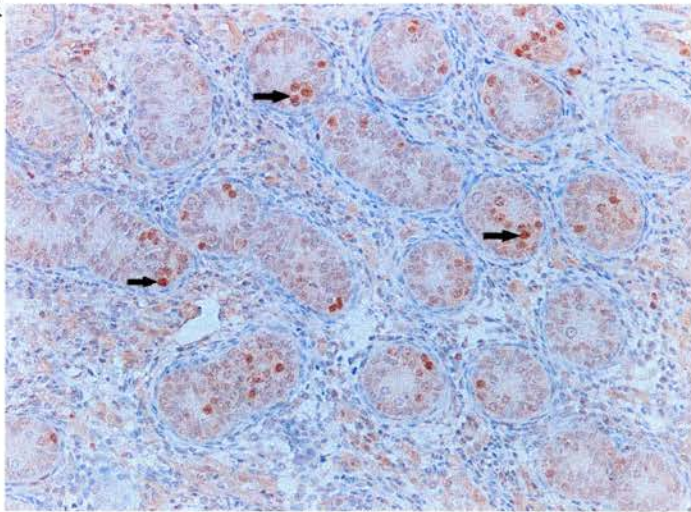
As the growth factor receptors previously identified are prominent during the prepubertal phase of testicular development, the aim was to identify a stem cell marker that persisted into this period. GFR- α 1 was expressed in occasional germ cells throughout all ages studied, in a pattern consistent with stem cell labelling (Fig 6.3). In younger animals, positive cells were noted to be located both basally and in the centre of the tubule away from the basement membrane, whereas from around 35 weeks onward germ cells expressing this antigen were exclusively basal.

To ascertain whether those germ cells expressing GFR- α 1 were actively proliferating, colocalization of this receptor was attempted with both PCNA and Ki67.

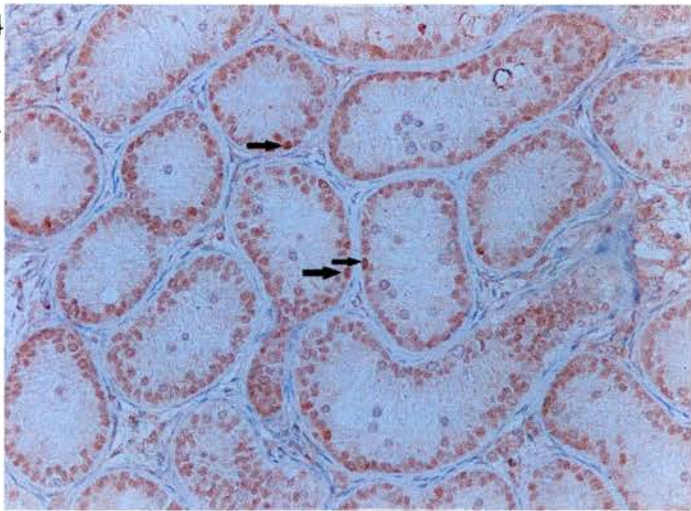
Immunofluorescent techniques to study GFR- α 1 (Fig 6.4) were problematic, as discussed above. Double staining using Fast Blue for detection of the proliferation marker was therefore also used, as shown in Figure 6.5. These studies showed that neither proliferation marker was expressed in the GFR- α 1 positive cells, suggesting that these cells were in a non-proliferative phase. However, fewer Ki67 positive cells were noted throughout the sections than would be expected from previous studies using DAB to visualise this marker, raising questions regarding the sensitivity of Fast Blue detection.

Figure 6.2 Immunoexpression of Oct-4 within (a) 1-day-old neonatal marmoset testis (b) 35-week-old marmoset testis (c) Adult marmoset testis

(a) Immunoexpression of Oct-4 in testis from a 1 day old control animal. Numerous germ cells appear to express this antigen at this age (arrowed) (20x magnification).



(b) Immunoexpression of Oct-4 in testis from a 35 week old control animal. Although a proportion of germ cells appear more intensely stained (arrowed), numerous other germ cells, and indeed Sertoli cells, are immunopositive, raising questions regarding the specificity of the primary antibody (20x magnification).



(c) Immunoexpression of Oct-4 in testis from an adult control animal. Although occasional germ cells appear more intensely stained (arrowed), as at 35 weeks of age non-specific staining exists, both in the seminiferous tubule and within the interstitium (10x magnification).

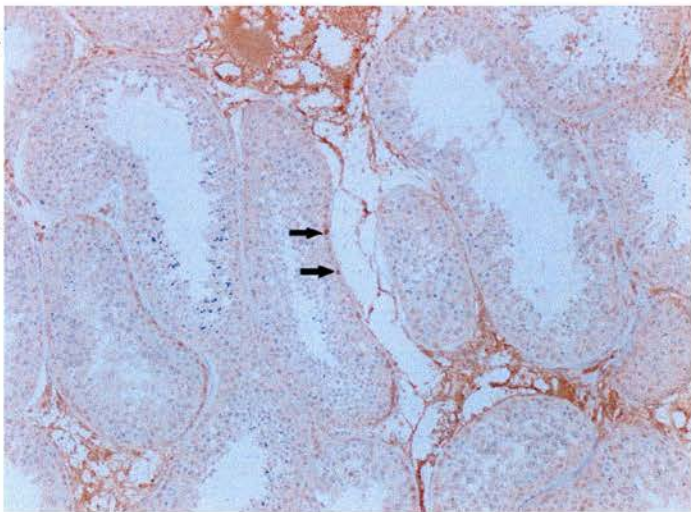
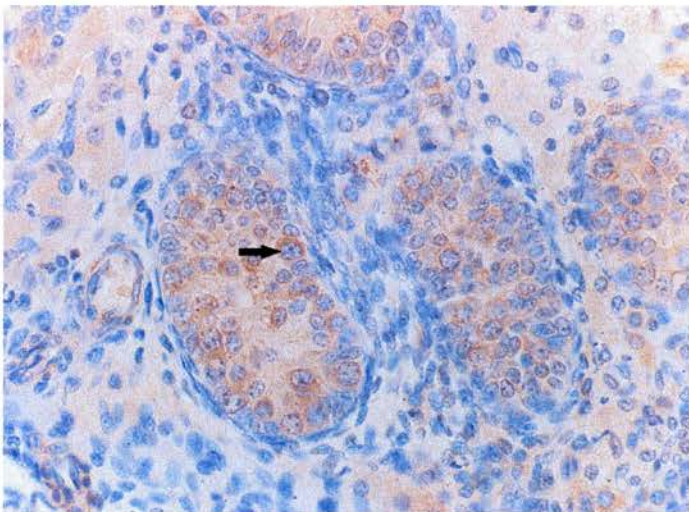
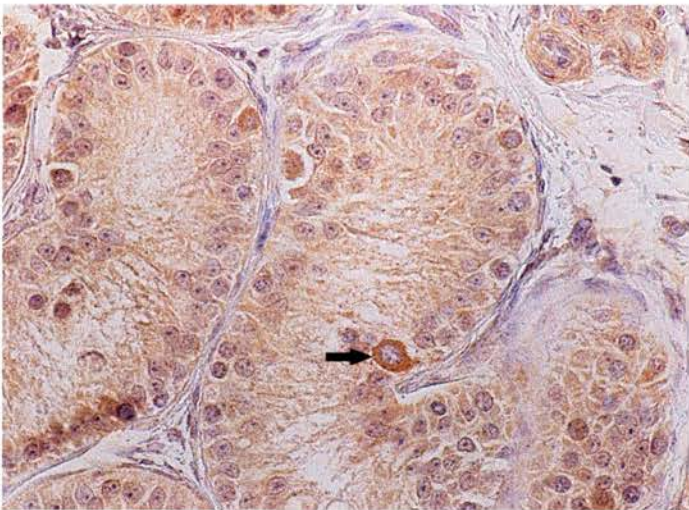


Figure 6.3 Immunoexpression of GFR- α 1 within (a) 2-day-old neonatal marmoset testis (b) 35-week-old marmoset testis (c) Adult marmoset testis

(a) Immunoexpression of GFR- α 1 in testis from a 2 day old control animal. Occasional germ cells express this receptor at this age (such as the one arrowed) (40x magnification).



(b) Immunoexpression of GFR- α 1 in testis from a 35 week old control animal. Occasional germ cells express this receptor at this age (such as the one arrowed) (40x magnification).



(c) Immunoexpression of GFR- α 1 in testis from an adult control animal. Occasional germ cells express this receptor at this age (such as those arrowed). Non-specific staining of elongate spermatid cytoplasm is also noted, as previously observed in immunostaining of marmoset testis at this age (40x magnification).

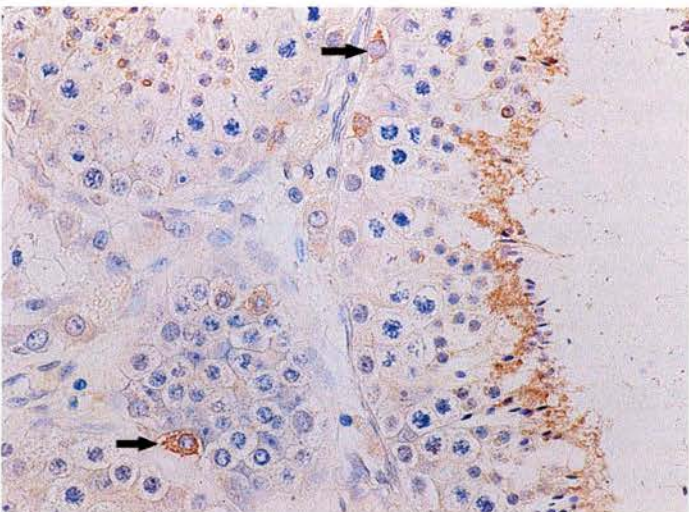
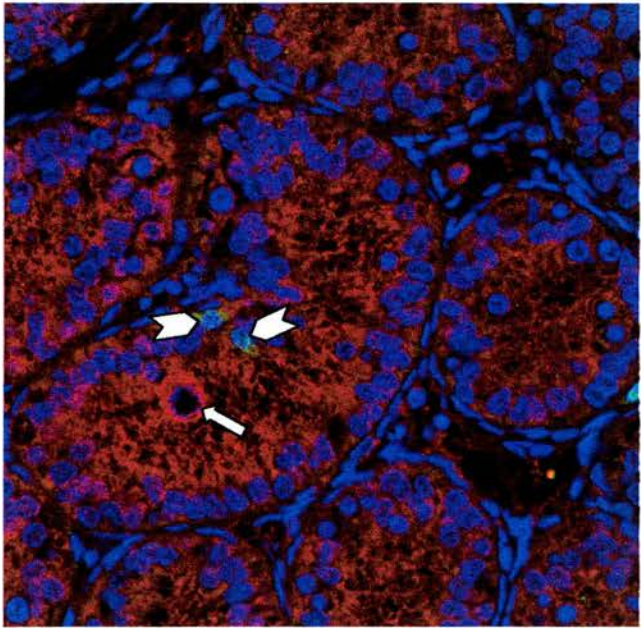


Figure 6.4 Localisation of the proliferation marker PCNA (green) with the GFR- α 1 receptor (red) in the marmoset testis using confocal microscopy
(a) prepubertal (b) adult
 (blue staining represents counterstain with propidium iodide)

(a) Immunoexpression of PCNA (green) and the GFR- α 1 receptor (red) in testis from an 18-24 week old control animal, using confocal microscopy. PCNA is localised to the nucleus of proliferating cells (indicated by arrowheads). The GFR- α 1 receptor is expressed in the cytoplasm of occasional germ cells (solid arrow). There is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time, although the excessive background staining makes interpretation difficult.



(b) Immunoexpression of PCNA (green) and the GFR- α 1 receptor (red) in testis from an adult control animal, using confocal microscopy. PCNA is localised to the nucleus of proliferating cells (indicated by arrowheads). The GFR- α 1 receptor is expressed in the cytoplasm of occasional germ cells (solid arrow). There is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time, although as above the excessive background staining makes interpretation difficult.

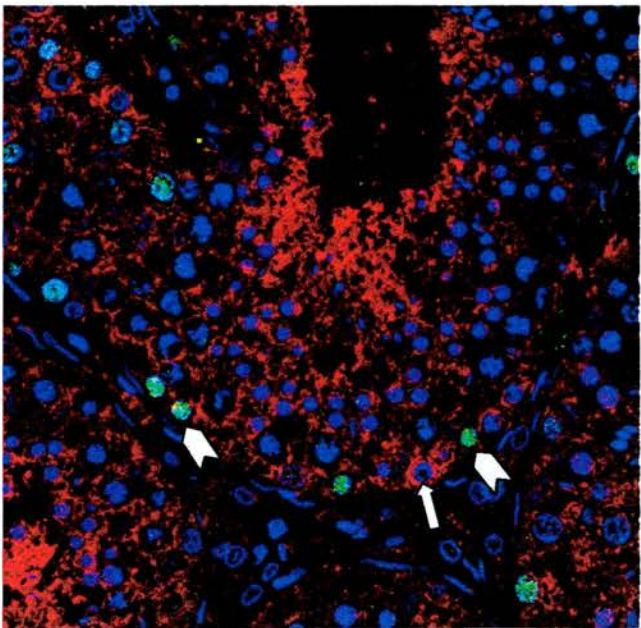
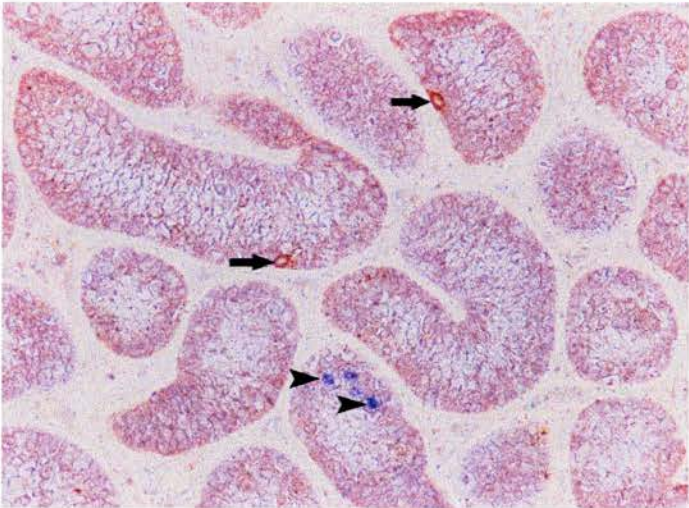


Figure 6.5 Localisation of the proliferation marker Ki67 (Fast Blue) with the GFR- α 1 receptor (DAB) in the marmoset testis at 35 weeks of age using double staining techniques

Immunoexpression of Ki67 together with the GFR- α 1 receptor in testis from a 35 week old control animal. Ki67 positive cells are stained with Fast Blue (indicated by arrowheads) whereas cells expressing the GFR- α 1 receptor are stained with DAB (indicated by solid arrows). There is no evidence of co-localisation of these antigens, in that no cell expresses both antigens at the same time, although fewer cells are immunopositive for Ki67 than expected from earlier studies, suggesting a lack of sensitivity with the detection process used for this antigen here.



6.4 Discussion

Within this part of the study, potential markers of stem cells in the marmoset testis were investigated, in order to ascertain their relationship with the growth factor receptors previously identified. Stem spermatogonia are capable of both differentiation into cells committed to more mature spermatogonia (ultimately resulting in spermatocytes and spermatozoa), and to self-renewal, in order to maintain the stem cell population. Studying this vital cell type is problematic, due to the small numbers of stem cells present within the testis, and the difficulty in identifying these cells based on morphological characteristics.

C-kit is the trans-membrane tyrosine kinase receptor for Stem Cell Factor (SCF) and is expressed in neonatal gonocytes in the rat (Orth *et al*, 1996). During embryogenesis, SCF binding is thought to play a role in the migration and proliferation of primordial germ cells (Orth *et al*, 1997). Based on rodent studies, SCF also plays a role in adult spermatogenesis, enhancing germ cell survival via a reduction in apoptosis (Yan *et al*, 2000). Sertoli cells produce SCF and the c-kit receptor is expressed on spermatogonia from differentiating type A spermatogonia through to pachytene spermatocytes (Vincent *et al*, 1998). However, expression of this receptor is not present on undifferentiated A spermatogonia.

Using the marmoset model, expression of the c-kit receptor has been demonstrated both on the membrane of neonatal germ cells and within the adult testis, in keeping with previous reports in the rat. However, no evidence of expression was found in the prepubertal testis.

Oct-4 is a transcription factor involved in regulation of pluripotency during normal development, and is detectable in embryonic stem and germ cells in the mouse (Pesce and Scholer, 2001). Immunohistochemical detection of this protein in human germ cell tumours suggests that Oct-4 represents a marker of pluripotent cells within the gonads as well as within germ cell tumours (Looijenga *et al*, 2003). It therefore represents a potentially useful avenue of investigation via which to study stem cells within the testis.

Using the marmoset model, immunoexpression of Oct-4 was detected in numerous germ cell nuclei during the neonatal period, although this finding was not consistent in all animals studied at this age. In addition, the pattern of expression of this protein during later stages of testicular development was difficult to elucidate because of the apparent poor specificity of staining observed. This problem persisted despite using alternative primary antibodies and attempts to optimise the immunohistochemical technique. Further investigation using Oct-4 as a stem cell marker was therefore not pursued, as reliable conclusions would be difficult to draw, particularly in the prepubertal age group.

As discussed previously, spermatogonia in the mouse are thought to express GFR- α 1 up until the A-aligned stage (Meng *et al*, 2000). It is hypothesised that GDNF, produced by Sertoli cells and acting via the GFR- α 1 receptor, promotes stem cell renewal and consequently acts to regulate the number of spermatogonial stem cells.

The immunoexpression of GFR- α 1 in the marmoset is certainly consistent with the pattern expected for a marker associated with germ cells during the early stages of

spermatogenesis. Within the neonatal period a number of germ cells expressed this receptor. During the prepubertal phase of testicular development the GFR- α 1 positive germ cells represent a very small proportion of the total cells present. In addition, the position of these cells within the seminiferous tubule undergoes a transition at around 18 to 24 weeks of age, from predominantly central, away from the basement membrane, to an exclusively basal location. In sections from postpubertal and adult animals, positive cells are noted on the basement membrane of occasional tubules.

Stem cells, by their nature, are very infrequent within the testis. It has been estimated that 10^4 germ cells isolated from adult rodent testes contain as few as 2 stem cells (Ogawa *et al*, 1997). The frequency of GFR- α 1 positive germ cells observed in the marmoset is in keeping with these representing stem cells. However, attempts to co-localize these cells with markers of proliferation suggest that germ cells expressing the GFR- α 1 receptor are not actively proliferating. This does not preclude these cells from being stem cells, as not all will be proliferating at any given time. In addition, the small number of GFR- α 1 positive cells present, and concerns regarding the sensitivity of Ki67 detection with Fast Blue, will also have impaired the ability to demonstrate simultaneous expression of GFR- α 1 with a proliferation marker. However, distinct conclusions regarding the window of GFR- α 1 expression and its functional significance in the marmoset are difficult to draw. In view of these uncertainties, further studies of this marker with the growth factor receptors previously identified were not performed.

At present the investigation of stem cells in many tissues, including the testis, is generating considerable interest within the scientific community. It is hoped that the rapidly expanding literature on this cell type, particularly within rodent models, will contribute to further studies of stem spermatogonia within the marmoset. This may include further investigation of the GFR- α 1 receptor, along with novel markers to reliably identify this cell population.

Chapter 7

Discussion

7.1 Conclusions

This study had two primary aims; firstly to investigate the hypothesis that prepubertal germ cell proliferation in the primate is gonadotrophin independent, and secondly, if this were to be confirmed, to investigate what factors do regulate their proliferation at this age. This information may subsequently lead to the development of strategies to protect fertility in prepubertal boys treated for cancer.

Firstly, as discussed previously, we can conclude that spermatogonial proliferation in the prepubertal marmoset is gonadotrophin independent. This explains, at least in part, the failure of gonadal protection strategies in both primates and humans based on suppression of the hypothalamic-pituitary-gonadal axis. The failure of these studies, despite the success observed in similar investigations using rats, also highlights the difficulty in extrapolating data from rodent models studying spermatogenesis to investigations involving primates. Significant differences exist between these species (Weinbauer and Nieschlag, 1989), particularly during the prepubertal phase of testicular development, and thus such studies must be interpreted in the appropriate context.

Secondly, we have identified a number of growth factors whose receptors are present on germ cells in the primate testis prior to puberty. Of the growth factor receptors studied here, the EGF receptor and the GFR- α 2 appear particularly prominent on a significant number of germ cells during the prepubertal period. However, the

limitations of the study and the technical difficulties encountered cannot exclude roles in germ cell regulation for the other growth factors investigated. In addition, those selected for study here do not represent an exhaustive list of those potentially involved, and indeed, additional and novel growth factors may be implicated in the future as having a role within the prepubertal primate testis.

Although these receptors have been identified on germ cells, the role their corresponding ligands may play in spermatogenesis is more difficult to ascertain. Because germ cells expressing either the EGF receptor or GFR- α 2 are not undergoing active proliferation, it is speculated that the corresponding growth factors, EGF and neurturin, regulate germ cell proliferation by inhibiting this process. These growth factors may inhibit intrinsic germ cell proliferation, or alternatively interfere with pathways that stimulate proliferative activity. This theory would appear to contradict the consensus view on the role of growth factors, but as discussed previously, there is some evidence for an inhibitory role in rodent models, at least with regards to EGF.

Confirming the precise roles these factors may have in spermatogenesis in the primate is problematic. Unlike studies involving rodents, where gene knockout models have contributed much to the literature, such studies are not possible, either practically or ethically, in primates. *In vitro* studies using cell lines in culture can provide useful information, but extrapolation to *in vivo* systems reduces the validity of the results obtained.

EGF and neurturin are not, however, unique to the primate. These factors have been identified in rodent models, and despite interspecies differences, their role in

spermatogenesis is likely to be similar. To date, confocal studies using these factors with markers of proliferation have not been previously reported, either in primates or rodent models. However, it would appear that these ligands perhaps have a more prominent role in the regulation of germ cell proliferation in primates, at least during the prepubertal period.

This prominence may simply be due to the more protracted prepubertal phase in primates as compared to rodents. Whilst this period lasts up until around 45 weeks of age in the marmoset and 12 years in humans, puberty commences within 15-20 days in the rat, and even sooner in the mouse. Thus although these growth factors may have an important role prior to puberty in rodents, they are unlikely to make as significant a contribution as in primates. In addition, because of its relatively short time period, investigation of the prepubertal rodent is more limited than in primate models. Following puberty, both FSH and testosterone become more dominant in the regulation of spermatogenesis (Sharpe, 1994). However, these growth factors may still have a role in the regulation of spermatogonial proliferation in adulthood, and indeed the present study provides no evidence that the regulatory processes in the postpubertal testis are different to those before puberty.

The significance of these growth factors in primates may also relate to their evolutionary development. Spermatogenesis in the rodent is a highly efficient and very organised process. This is in complete contrast to the inefficiency and poor organisation found in testes from a number of primates. It is possible that 'higher' mammals have lost this efficiency because they do not need to produce large numbers of offspring in order to propagate their species. Indeed, fewer offspring may actually

improve overall survival because of the more complex developmental requirements of younger primates. Rodents, on the other hand, must produce large numbers of offspring to continue the gene line. Therefore, one may speculate that spermatogenesis in primates is perhaps regulated by more 'primitive' mechanisms, such as the growth factors identified in this study.

7.2 Limitations of Immunohistochemistry

Analyses of the immunoexpression of the receptors and their ligands discussed highlight a number of the limitations of immunohistochemistry. Detection of proteins via immunohistochemical methods is reliant on both the specificity of the primary antibody and the sensitivity of the method of detection. Both these steps in the procedure can be validated by the use of positive controls, using tissue that is known to have positive expression of the antigen under investigation. However, many primary antibodies used are species specific and therefore require appropriate tissue from the same species to be obtained, which can be problematic.

However, with the exception of investigation of the GM-CSF receptor, the major problem encountered in this immunohistochemical study was not lack of detection, but of significant background staining in the sections studied. In this circumstance a negative control is essential, in which primary antibody is omitted from one section. This ascertains the contribution of the secondary antibody to the staining pattern observed. Within the marmoset tissue studied here, these controls were negative, suggesting that the secondary antibody was not the source of background staining but that the primary antibody was not sufficiently specific. The use of alternative primary

antibodies, along with various techniques to optimise immunohistochemical detection, can be beneficial, but the interpretation of certain antigens studied here is limited.

The problem of background staining is compounded by the use of immunofluorescence. Tyramide enhancement is a very sensitive detection system, but the primary antibodies used are the same as for conventional immunohistochemistry, and therefore have the same specificity. Consequently, significant background can impair the analysis and interpretation of these studies.

Despite these limitations, immunohistochemistry remains the only viable approach to investigating the presence of proteins in a subset of cells within a multicellular tissue. In addition, other scientific techniques that are applicable to rodent models are not possible in primate research, as discussed previously. As such, these studies contribute significantly to our current understanding of the marmoset testis, and will help direct future investigation of the regulation of germ cell proliferation in this species.

7.3 Future Studies

Future studies must initially be directed towards confirming the precise roles the factors identified here have within the prepubertal primate testis, and more specifically investigating the hypothesis that EGF and neurturin act in a paracrine manner to inhibit spermatogonial proliferation. The use of rodent models, particularly prepubertal animals, will certainly contribute to this investigation. However, the marmoset is the most appropriate animal model in which to take these studies forward.

Because gene knockout models, *in vitro* studies and other potentially useful techniques such as RNA interference (RNAi), which can be used to 'silence' certain genes, are not possible in primate species, alternative modes of investigation are required. This may involve *in vivo* studies, with administration of either the growth factor itself or an antagonist into the animal model and subsequent assessment of the proliferation rate of germ cells. Administration could either be systemic or indeed unilateral via the intratesticular route, and the use of co-twins will be invaluable for analysis of the effect of any intervention. These studies will then assist in the design of protective strategies to protect the testis from cytotoxic damage. In order for this to be achieved the proliferation rate of spermatogonia, and particularly stem cells, must be reduced, in order to reduce their susceptibility to cytotoxic agents. If EGF and neurturin work in an inhibitory manner then supplementation of these growth factors may achieve this result. However, if these growth factors are only inhibitory within a specific window of concentration, beyond which stimulation of proliferation occurs, as has been suggested for EGF, then therapeutic intervention may be more difficult to realise.

Should these studies prove that manipulation of these growth factors could protect the prepubertal primate testis from cytotoxic damage, two further issues need to be addressed before clinical application of this type of procedure.

Firstly, the relevance of these growth factors in the human testis must be established. Although the marmoset is an excellent animal model in which to study spermatogenesis, and certainly more representative than rodent models, differences

may exist in the human that are important to identify. Studies are currently underway on human testicular tissue, investigating the immunoexpression of the growth factor receptors identified in the marmoset. However, problems with the quality and variability of the tissue affect the interpretation of such results. In addition, obtaining human testicular tissue for research purposes presents particular ethical difficulties.

Secondly, growth factors have numerous essential physiological roles outwith the testis. Thus any manipulation of these factors must not cause any unacceptable adverse effects. This may be particularly important both during childhood, when normal growth and development are at a critical stage, and in patients with cancer, due to the possibility of growth factors acting on malignant cells.

Transgenic mice with overexpression of EGF exhibit intrauterine growth retardation, with birth weights half that of normal littermates (Wong *et al*, 2000). Administration of EGF to newborn mice also results in growth retardation and impairs the appearance of several neurobehavioral signs of maturation (Calamandrei and Alleva, 1989).

GDNF and its related ligands are involved in neuronal survival and renal development, which may potentially be affected by manipulation of these factors. In addition, mutations of the RET signalling system cause several human diseases, including papillary thyroid carcinoma, multiple endocrine neoplasia types 2A and 2B, and Hirschsprung's disease (Takahashi, 2001).

The expression of the EGF receptor has been demonstrated in a variety of human tumours, particularly those seen in the adult population such as breast cancer (Normanno *et al*, 2003). Within these tissues, EGF may play a role in regulating the

proliferation and survival of malignant cells. Indeed, anti-EGF receptor antibodies and EGF receptor tyrosine kinase inhibitors have been developed as novel strategies in the treatment of these tumours (Baselga, 2001). Transgenic mice with overexpression of GDNF develop malignant testicular tumours, which mimic seminomas (Meng *et al*, 2001b).

Manipulation of either of these growth factors therefore has the theoretical potential to interfere with a child's normal growth and development, or worsen their malignant disease. These adverse effects are clearly unacceptable, and as such any intervention to protect fertility in this manner must be of proven safety before clinical application, both in the short and long term. Local treatment with intratesticular administration is likely to be less problematic than systemic administration, but damage to the testes must be minimised, and not outweigh any potential benefits.

A further issue that would need to be addressed, should fertility protection in this manner become feasible, is the time required to achieve inhibition of spermatogonial proliferation prior to commencing cytotoxic treatment. Suppression of the hypothalamic-pituitary-gonadal axis in rodent models requires several weeks of hormonal intervention before the gonadotoxic insult (Kurdoglu *et al*, 1994; Kangasniemi *et al*, 1995). This delay is unsatisfactory for the majority of cancer patients, who generally must start treatment soon after diagnosis. It is hoped that further investigation of these growth factors in the prepubertal testis will indicate the likely time period necessary for adequate testicular suppression.

7.4 Ethical and Legal Issues

Manipulation of factors that regulate spermatogonial proliferation in order to improve future spermatogenesis, along with other techniques of fertility preservation such as harvesting gonadal tissue for future use, are exciting prospects that provide hope for children with cancer. Although there are still many scientific and technical issues to resolve, this technology also raises a number of important ethical and legal issues, which must be addressed before these procedures are utilised in a clinical setting.

Of prime importance, when considering options for future fertility following childhood cancer treatment, must be that any decision is taken in the child's best interests. Thus the advantages of any intervention, or of an active decision not to intervene, must outweigh any disadvantages, both in the short and long term. Attempts to preserve fertility must not raise unrealistic expectations, and must not have undue adverse effects in either the patient or any subsequent offspring.

Comparing potential benefits with long-term risks is particularly problematic in this situation. The effectiveness of therapeutic intervention is still unknown at present, and it will be many years until expertise has improved sufficiently to assess it realistically. However, unless these techniques are considered and appropriate methods offered now, the opportunity of fertility preservation will be missed. Deleterious effects will also take many years to fully evaluate, particularly with respect to future progeny. Thus fertility preservation must be considered in the context of clinical benefit within the management of childhood cancer, and also in the context of ongoing research. Valid consent to perform these procedures is therefore both a legal and ethical requirement.

For consent to be valid it must be informed, obtained voluntarily and given by a competent person. Legal competence to consent requires that the individual giving it is able to understand the information given, believes it applies to them, retains it, and uses it to make an informed choice. In view of the complexity of the issues surrounding fertility preservation, the anxieties of both patients and their families at the time of diagnosis, and the limited time for discussion due to the urgency of commencing treatment, the validity of such consent may be impaired.

The issue of valid consent is further complicated by the age of the patient involved and their degree of understanding of the issues being discussed. Young persons over the age of 16 years in Scotland, and 18 years in England and Wales may consent to treatment under the Family Law Reform Act (1969). Otherwise consent is obtained by proxy, from a parent or legal guardian. Younger children may give valid consent if they demonstrate sufficient understanding and intelligence to enable them to make an informed decision, so called "Gillick competence" (*Gillick v West Norfolk and Wisbech Area Authority*, 1985). However, with respect to the storage and future use of gametes, consent by proxy is specifically excluded by the Human Fertilisation and Embryology Act (1990). Thus parents, or legal guardians, cannot give consent on behalf of the child. Immature germ cells, however, are not within the HFEA definition of a gamete as they are unable to take part in fertilisation. Therefore these cells could be harvested with parental consent, if the procedure was in the child's best interests. If this immature material were subsequently matured to produce gametes this tissue would then fall under the jurisdiction of the HFEA.

Consent in situations such as this should be viewed as a dynamic, continual process that is adapted as new information becomes available. Indeed, many of the difficulties discussed above may be alleviated by obtaining consent in different stages (Grundy *et al*, 2001). The first stage of consent would be for the harvest and storage of the gonadal tissue. The second stage, at a later date, would involve consent for the use of stored germ cell material for both fertilisation and research. In addition it is important to consider what should happen to stored gonadal tissue in the event of the child's death. Whilst some would advocate destruction of the tissue in this situation, others have suggested allowing the parents to consent for the tissue to be used for research purposes (Wallace and Walker, 2001).

These issues must be addressed in order that new techniques are adequately regulated. Following extensive, collaborative discussion within a multidisciplinary setting, a number of recommendations have been suggested (Wallace and Walker, 2001; British Fertility Society, 2003). These include ongoing, structured research with centralization of data and rapid dissemination of results, a rigorous review of procedures and development of the process of obtaining informed consent. This will ensure that children with cancer have a realistic and safe prospect for fertility in the future.

7.5 Concluding Remarks

Infertility can be a major long-term side effect following treatment for childhood cancer, and will become increasingly important as greater numbers of children survive into adulthood. It is therefore imperative to consider ways of protecting or restoring fertility at an early stage.

At present there is nothing to offer the prepubertal boy at risk of infertility. Protection of fertility during gonadotoxic therapy by inducing testicular quiescence is a potentially useful and acceptable mode of intervention for these patients. Although there are numerous issues to be addressed before these techniques become reality, the identification of factors that regulate spermatogonial proliferation in the prepubertal testis contributes both to our understanding of testicular physiology, and to devising such intervention in this patient group. Further studies into this area may therefore offer genuine hope for childhood cancer survivors at risk of infertility in the future.

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Publications arising from this thesis

- Sharpe RM, Fraser HM, Brougham MF, McKinnell C, Morris KD, Kelnar CJ, Wallace WH, Walker M. Role of the neonatal period of pituitary-testicular activity in germ cell proliferation and differentiation in the primate testis. *Hum Reprod* 2003; 18: 2110-2117.
- Brougham MFH, Kelnar CJH, Sharpe RM, Wallace WHB. Male Fertility following Childhood Cancer: Current Concepts and Future Therapies. *Asian Journal of Andrology* 2003; 5 (4): 325-337
- Wallace WHB, Brougham MFH. Subfertility in adolescents with cancer: Who is at risk and what can be done? In: Eden, Barr, Bleyer and Whiteson (eds). *Cancer and the Adolescent: Second Edition* (2005). Blackwell Publishing (BMJ Books) pp133-154
- Brougham MFH, Sharpe RM, McKinnell C, Evans AE, Kelnar CJH, Wallace WHB. The regulation of germ cell proliferation in the prepubertal marmoset: towards a strategy for fertility preservation in boys treated for cancer. Manuscript in progress.

Abstracts arising from this thesis

- Brougham MFH, Sharpe RM, McKinnell C, Kelnar CJH, Wallace WHB. An investigation of spermatogonial proliferation in the prepubertal marmoset testis: towards a strategy for fertility preservation in boys treated for cancer. Abstract submitted to the 35th Congress of the International Society of Paediatric Oncology, and accepted as an oral presentation.
- Brougham MFH, Sharpe RM, McKinnell C, Wallace WHB, Kelnar CJH. Identification of stem spermatogonia in the prepubertal marmoset testis: towards a strategy for fertility preservation in boys treated for cancer. Abstract submitted to the 43rd annual meeting of the European Society of Paediatric Endocrinology, and accepted as a poster presentation.

Appendix A- Cell quantification data for Chapter 4

MAGE (Figs 4.2 and 4.4)

		Mean ratio of stained to unstained cells	Standard deviation
35-week-old co-twins <i>n</i> =4	Control	0.504	0.097
	Treated	0.574	0.179
4-week-old co-twins <i>n</i> =4	Control	0.171	0.060
	Treated	0.132	0.021

Ki67 (Fig 4.7)

		Mean ratio of stained to unstained cells	Standard deviation
35-week-old co-twins <i>n</i> =4	Control	0.0145	0.0044
	Treated	0.0138	0.0031

Histone H3 (Fig 4.8)

		Mean ratio of stained to unstained germ cells	Standard deviation
35-week-old co-twins <i>n</i> =4	Control	0.0055	0.000577
	Treated	0.00713	0.005513
All animals (singletons and co-twins)	Control <i>n</i> =7	0.01133	0.004726
	Treated <i>n</i> =9	0.0085	0.012757

Appendix B Quantification of GFR-α2 immuno-positive cells (Figure 5.3)

Co-twin pair		Total germ cell number per testis (x10 ⁵)		Ratio of stained:unstained germ cells
		Stained	Unstained	
i	Control	4.533982	5.500566	0.824
	Treated	6.853556	6.770205	1.012
ii	Control	4.329006	10.18262	0.425
	Treated	4.787974	4.215133	1.136
iii	Control	6.349825	6.935125	0.916
	Treated	2.985234	5.854137	0.510
iv	Control	4.537608	3.99696	1.135
	Treated	3.892298	6.217289	0.626